Analytical Chemistry of Hydroxylated Metabolites of PCBs and other Halogenated Phenolic Compounds in Blood and Their Relationship to Thyroid Hormone and Retinol Homeostasis in Humans and Polar Bears

by

Courtney Douglas Sandau

A thesis submitted to

the Faculty of Graduate Studies and Research

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

Carleton University

Ottawa, Ontario

December 15, 2000

Abstract

Metabolites of xenobiotic compounds have been identified and monitored but were always thought to be excretion products and regarded as having little toxicological significance. Polychlorinated biphenyl (PCB) metabolites were discovered almost immediately after the discovery of PCBs but it was not until 20 years later, their significance was determined. Analytical methodology for hydroxylated metabolites of PCBs (OH-PCBs) was established for blood and plasma and applied to the analysis of polar bears and human plasma. During method development, a new chlorinated phenolic compound was discovered and its identity determined as a likely metabolite of octachlorostyrene. Polar bear plasma from Resolute Bay, Canada and Svalbard, Norway were found to have the highest levels of OH-PCBs of any species analyzed. Concentrations of OH-PCBs often exceeded the concentration of PCBs themselves making OH-PCBs the most abundant class of contaminants in polar bear plasma. In polar bears, OH-PCBs were found to be positively associated with plasma retinol concentrations ($r=+0.31$, $p=0.02$, $n=57$) and negatively associated with the plasma free T4 index ($r=-0.44$, $p<0.001$, $n=55$) possibly indicating disruption of retinol and thyroid hormone homeostasis.

OH-PCBs and other halogenated phenolic compounds were also analyzed in Inuit whole blood and human umbilical cord plasma for the first time. Patterns and concentrations were determined for each. Pentachlorophenol was concluded to be one of the most important compounds in the phenolic compound fraction due to its high concentration in all human blood samples analyzed.

List of Publications Associated with Dissertation

Refereed Journals

C.D. Sandau, I.A.T.M. Meerts, R.J. Letcher, A. McAlees, B. Chittim, A. Brouwer, R.J. Norstrom, 2000. Identification of 4-hydroxy-heptachlorostyrene in polar bear plasma and its binding affinity to transthyretin: a metabolite of octachlorostyrene? *Environmental Science & Technology* 34 (18) p. 3871-3877.

C.D. Sandau, P. Ayotte, É. Dewailly, J. Duffe, R.J. Norstrom, 2000. Analysis of hydroxylated metabolites of PCBs and other chlorinated phenolic compounds in whole blood from Canadian Inuit. *Environmental Health Perspectives*, Vol 108, No. 7, p. 611-616.

C.D. Sandau, P. Ayotte, É. Dewailly, J. Duffe, R.J. Norstrom, (in preparation) PCP and hydroxylated metabolites of PCBs in umbilical cord plasma from coastal regions of Quebec. *Environmental Health Perspectives*.

Extended Conference Abstracts

C.D. Sandau, M. Ramsay, R.J. Norstrom, 2000. Implication of hydroxylated metabolites of PCBs and other halogenated phenolic compounds as endocrine disruptors in polar bears. *Enviro*Analysis 2000, Proceedings of the third biennial international conference on chemical measurement and monitoring of the environment. May 8-11, 2000.

S.C. Newson, C.D. Sandau, J.E. Elliott, S.B. Brown, R.J. Norstrom, 2000. PCBs and hydroxylated metabolites in bald eagle plasma – Comparison to thyroid hormone and retinol levels. *Enviro*Analysis 2000, Proceedings of the third biennial international conference on chemical measurement and monitoring of the environment. May 8-11, 2000.

C.D. Sandau, P. Ayotte, É. Dewailly, Å. Bergman, E. Klasson-Wehler, R.J. Norstrom, 1998. Analysis of hydroxylated metabolites of PCBs (OH-PCBs) in whole blood from Canadian Inuit. *Dioxin '98, Organohalogen Compounds*, Stockholm, Sweden. Vol. 38, p.29-32.

C.D. Sandau, R.J. Norstrom, 1998. Analysis of hydroxylated metabolites of PCBs (OH-PCBs) in polar bear plasma and human whole blood. *Enviro*Analysis'98, Proceedings of the second biennial international conference on chemical measurement and monitoring of the environment. p. 405-410.

C.D. Sandau, R.J. Norstrom, 1996. Comparison of methylation methods for the determination of hydroxy-PCBs and preliminary results for polar bear blood plasma.

Dioxin '96, Organohalogen Compounds, University of Amsterdam Press, Amsterdam, The Netherlands. Vol. 29, p. 412-417.
Conference proceedings

C.D. Sandau, P. Ayotte, É. Dewailly, R.J. Norstrom. Hydroxylated Metabolites of PCBs (OH-PCBs) in human blood. PCB Workshop, Lexington, Kentucky April 9-12, 2000 (Poster No. 59).

C.D. Sandau, S. Newson, J.E. Elliott, R.J. Norstrom, 1999. PCBs and their hydroxylated metabolites in Bald Eagle plasma and comparison to thyroid hormone levels. *SETAC 1999.* (Poster – PTA161)

K. G. Drouillard, C.D. Sandau, R.J. Norstrom, 1998. Quantifying plasma lipids in avians using gravimetric, colorimetric and enzymatic techniques. *SETAC 1998.* (Poster – PTA007)

Acknowledgements

I would like to start by thanking my supervisor, Ross Norstrom, for giving me the opportunity to do this research. I would also like to thank all the people at the National Wildlife Research Centre (NWRC, Hull, Quebec) for providing a fun working environment and for all their help and support for my research projects. Special thanks to Sheryl Tittlemier, Craig Hebert, Aaron Fisk, Ken Drouillard and Rob Letcher for providing the laughs that made the time go so quickly.

I thank my parents for their support and encouragement, which enabled me to get to the end. Dad, summers at the Bugaboos digging ditches in mud and rock and being told to "climb down that sewer and unplug that" was all the encouragement that I needed! I would also thank my second set of parents (Barb and Doug) who always supported my family and I in everything we did.

The biggest support came from my wonderful wife, Connie. Her proof reading helped my writing and her patience allowed me to work the endless hours needed to complete this thesis. I would also like to acknowledge my daughter, Brianna, for being one of the only people that could take my mind off work and could always bring a smile to my face.

I dedicate this thesis to the memory of my grandfather who always thought I could accomplish something special with my life. I also dedicate this to my wife and family. Thank you for your support. I love you.

Table of Contents

List of Figures

List of Figures (continued)

List of Figures (continued)

List of Figures (continued)

List of Figures (continued)

List of Tables

List of Tables (continued)

List of Tables (continued)

List of Appendix Tables

Glossary of Acronyms

4-OH-HpCS	4-hydroxy-heptachlorostyrene
ARRF	Apparent relative response factor
CB#	Chlorobiphenyl, IUPAC numbering according to Ballschmiter and Zell (1) with modifications by Guitart *et al.* (2)
CC	Correlation coefficient
CHCs	Chlorinated hydrocarbon contaminants
CHL	chlordanes
CV	Coefficient of variation
CYP	Cytochrome P450 monooxygenase enzyme
DCM	Dichloromethane
DDD	*Bis*-2,2-(4-chlorophenyl)-1,1-dichloroethane
DDE	*Bis*-2,2-(4-chlorophenyl)-1,1-dichloroethene
DDT	*Bis*-2,2-(4-chlorophenyl)-1,1-trichloroethane
DLs	Detection limits
ECD	Electron capture detector
ECNI	Electron capture negative ionization
eV	Electron volts
FT4	Free thyroxine
GC-ECD	Gas chromatography - electron capture detection
GC-ECNI-MS	Gas chromatography - electron capture negative ion mass spectrometry
GC-EI-MS	Gas chromatography - electron impact mass spectrometry
HCB	Hexachlorobenzene
HCH	Hexachlorocyclohexane
i.d.	Internal diameter
IDL	Instrument detection limit
IPA	Ion-pair alkylation
IS	Internal standard
MeOH	Methanol
MTBE	Methyl-*tert*-butyl ether
OCS	Octachlorostyrene
PAGE	Polyacrylamide gel electrophoresis
PBP	Pentabromophenol
PBQ method	Polar bear quantitation method
PCA	Principal component analysis
PCB	Polychlorinated biphenyl
PCBs	Polychlorinated biphenyls
PCP	Pentachlorophenol
PCs	Principal component
PnClBz	Pentachlorobenzene
ppb	Parts per billion

ppt	Parts per trillion
PS	Performance standard
RBA	Relative binding affinities
RI	Retention index
RRF	Relative response factor
S/N	Signal-to-noise ratio
SIM	Selective ion monitoring
STs	Sulfotransferases
T3	Triiodothyronine
T4	Tetraiodothyronine Thyroxine
TBG	Thyroxine Binding Globulin
TeClBz	Tetrachlorobenzene
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone Thyrotropin
TT4	Total thyroxine
TTR	Transthyretin, Prealbumin
UDP-GTs	Uridine diphosphate-glucuronosyltransterases

Chapter 1. Introduction

Rachel Carson's book "Silent Spring" (1962) prompted extensive research of persistent environmental contaminants. This book publicized the effects of contaminants, primarily dichlorodiphenyltrichloroethane (DDT), on biota. The research that followed on DDT led to the discovery of polychlorinated biphenyls (PCBs) and this brought about the discovery of other organochlorine compounds. After almost 40 years of research, effects of environmental contaminants are still not well understood and even more compounds continue to be discovered. This thesis is based on new aspects of as well as the old problems of PCBs.

The "old" problem refers to the fact that PCBs were discovered 34 years ago and PCBs have been banned in most industrialized nations for the past 20 years. Immediately following the ban, environmental levels dropped dramatically, but at present, concentrations seem to be stabilizing. These trends have been demonstrated in Great Lake herring gulls (3). This stabilization indicates that there will be no major decline in the coming years. Even using conservative concentration estimates, present day levels are thought to be high enough to exude toxicological effects, such as those affecting thyroid hormone homeostasis (4). The familiar compounds - PCBs, chlordanes, hexachlorocyclohexanes, chlorinated benzenes, DDT and its metabolites have been monitored continuously for the last 30 years. These same contaminants are analyzed in blood samples in the current thesis.

The "new" aspect investigates the latest discoveries on possible links of PCBs and their effects on biota at the molecular level. Since the early 1990s, research has

exploded on the effects of contaminants on the endocrine system. This type of research is referred to, in popular terms, as endocrine disruption. Through a better understanding of cellular and molecular processes and the modes of action of contaminants on cellular processes, the discovery of these important mechanistic effects need to be applied at the organism level. Showing competitive binding in a hormone assay in a test tube does not imply that the whole organism is at risk for showing that same effect.

This thesis is concerned with the hydroxylated metabolites of PCBs, along with other halogenated phenolic compounds, and their possible deleterious *in vivo* effects on retinol and thyroid hormone homeostasis. Hydroxylated metabolites were recently implicated as important contaminants when it was discovered that they can selectively accumulate in plasma due to affinity for transthyretin, which can be linked to their structural similarities to thyroid hormones (5). Multiple metabolites can be formed from individual PCBs so the complicated task of identifying retained compounds is underway (6). This thesis describes the development of a method of analysis, the identification of numerous metabolites and other phenolic compounds, and the patterns of OH-PCBs using plasma from various species, including humans. Attempts to relate concentrations of contaminants, including the phenolic compounds to biological measures to assess the overall effects of contaminants on those measures were also undertaken. Thus, the hypothesis is that hydroxylated metabolites of PCBs are responsible for some of the toxic effects in animals exposed to PCBs. The metabolites which accumulate in animals and are responsible for these effects are structurally similar to thyroid hormones, T3 and T4 and the metabolites' mode of

action is through their ability to bind to thyroid hormone transport proteins and/or receptors.

1.1. Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) are a group of chlorinated aromatic hydrocarbons, which have been manufactured industrially since 1929 (7). PCB mixtures are produced as technical mixtures by chlorinating biphenyl with anhydrous chlorine using ferric chloride or iron filings as a catalyst (7). The mixtures are characterized by the percentage weight chlorine (e.g. Aroclor 1248 - 48% chlorine by weight) and were sold under trade names such as Aroclor (USA), Kanechlor (Japan), Clophen (Germany), Phenoclor (France), and Sovol (former USSR).

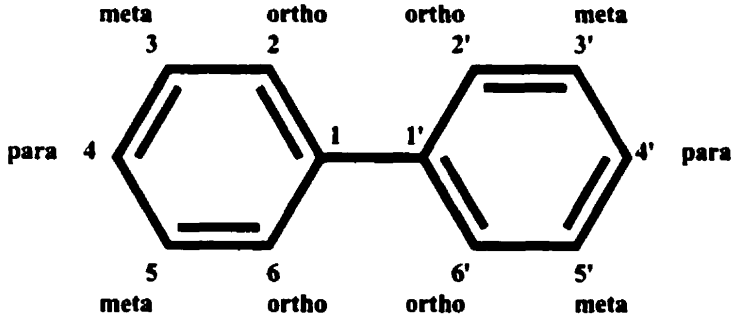


Figure 1.1 - The chemical structure of polychlorinated biphenyls with numbering and positions labeled.

Of the 209 possible congeners that can form by chlorinating biphenyl, only about 120 are present in commercial mixtures (8). In general, chlorine substitutions on either of the biphenyl rings differ only by one or two chlorines (9). PCBs have been assigned a systematic numbering scheme following International Union of Pure and Applied Chemistry (IUPAC) guidelines for identification by Ballschmiter (1),

which was later modified by Guitart *et al.* (2). The numbering scheme is demonstrated in Figure 1.1 and structures for all 209 congeners are given in Appendix Tables 1 and 2. Appendix Table 2 also gives the PCB nomenclature according to substitution in each ring to make it easier to visualize structures from the PCB number of a given chlorination pattern.

From the 1930s to the mid-1970s, PCBs were used extensively. Some of the main uses included heat transfer fluids (in heat exchangers and hydraulic fluids), organic diluents (in plastics, paints, adhesives, lubricants, sealants), pesticide extenders, flame retardants, cutting oils, dielectric fluids for transformers and capacitors and even as a component of carbonless copy paper (7). Some of the main desirable properties of PCBs were their chemical stability, insulating properties and high temperature resistance. Due to careless disposal practices, accidents and leakage, PCBs have found their way into the environment. PCBs were widely used and unnoticed in the environment for 37 years until discovered by Sören Jensen in 1966 (10, 11).

Through processes such as 'global distillation' and 'cold condensation', compounds with low volatility, such as PCBs, undergo long-range atmospheric transport (12) allowing them to be transported to remote regions and become ubiquitous environmental contaminants. Due to technological improvements in analytical chemistry, detection limits have dropped dramatically in the last 20 years allowing PCB quantitation in virtually every known matrix. Since Jensen's discovery, PCBs have been quantitated in Antarctic Ocean samples (13), deep Atlantic Ocean sediments (14), air samples (15), phytoplankton (16) and Arctic marine mammals

(17). The widespread detection of PCBs clearly demonstrates their global distribution and persistence.

The properties that made PCBs desirable in industry were their thermal stability, resistance to acids and bases and stability to conditions of oxidation and hydrolysis. The resistance of PCBs to chemical reactions combined with their lipophilicity has facilitated the bioaccumulation and biomagnification of PCBs up through the food chain. Depending on the PCB structure, this has resulted in high, sustained levels of PCBs in the top-predators (18) - including humans (19).

The industrially useful properties of PCBs do not preclude them from biotransformation. PCBs can be biotransformed by the cytochrome P450 monooxygenases (CYP). This diverse class of enzymes is mainly responsible for the insertion of oxygen into both endogenous and exogenous compounds. Oxygen insertion can eventually lead to hydroxylated metabolites which can be converted into more water-soluble compounds that generally facilitates excretion or further conjugation and excretion.

CYP enzymes are membrane bound enzymes and are located in every type of cell except red blood cells and skeletal muscle cells (20). The highest concentration of these types of enzymes is in the liver. CYP enzymes are structurally diverse and it is estimated that mammals have anywhere from 60 to over 200 forms of CYP. A comprehensive review of the forms and functions of CYP enzymes is supplied by Ortiz de Montellano (21). Of the many possible isoforms of CYPs, the subfamilies CYP1A and 2B have been extensively studied in regards to their interactions in the hydroxylation of PCBs.

Biotransformation rates are related to the structure of PCBs and are generally slow for most PCBs that accumulate in biota. Biotransformation rates are also species specific as species have varying amounts and types of CYP enzymes (22-24). Levels of enzymes can be increased with exposure to inducing agents and generally, inducing agents are also substrates.

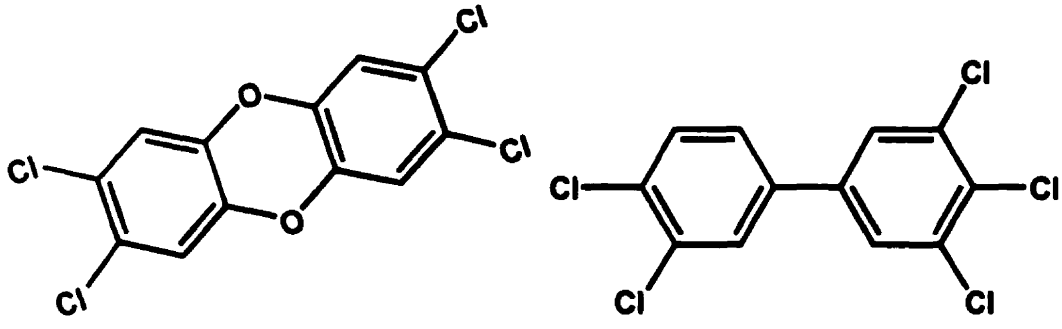
1.2. Induction system of the metabolizing enzymes

In order to understand hydroxylated metabolites of PCBs and their effects, the mechanism of oxidation that leads to their formation must be elucidated. The biotransformation of PCBs takes place by CYP mediated oxidation, therefore, the mechanism of induction of these enzymes is important.

The induction of hepatic cytochrome CYPs and diverse CYP-dependent monooxygenases is a sensitive indicator of PCB exposure that has been observed in multiple species (25). PCBs have been historically classified into three types of CYP inducers - phenobarbital (PB)-type, 3-methylcholanthrene (MC)-type and mixed-type (both PB and MC type). The different types of induction facilitated by PCBs are dependent on the different chlorine substitution pattern on each of the biphenyl rings (25-28).

PCBs that cause "PB-type" induction contain at least one chlorine in the ortho position of the biphenyl ring, which reduces free rotation of the biphenyl rings. This hinders the ability of the PCB in assuming a planar biphenyl configuration. The most active PCBs that act as PB-type inducers contain at least two ortho and two para chlorine substituents (29). However, no comprehensive structure-activity rules have

been developed for PB-type inducers and their induction mechanism remains to be determined. PB-type inducers are thought to have a similar induction mechanism as MC-inducers through the recently described barbiturate-responsive regulatory sequence (Barbie-box) where PB derepresses CYP gene expression resulting in increased transcription for CYP2B type enzymes in mammals (30). Another induction mechanism, involving the constitutive androstane receptor (CAR) is also thought to play a role in the induction of CYP2B enzymes. The CAR receptor is generally occupied by endogenous androstane like steroids. Upon exposure to PB-type inducers, binding of the steroids to the CAR receptor is inhibited leading to derepressed receptor activity resulting in subsequent gene transcription (31, 32). Little is known about other steps in the molecular mechanisms of CYP2B induction, but current models are summarized elsewhere (33-35) and will not be discussed further.



TCDD CB126

Figure 1.2 - Examples of potent CYP1A inducers - TCDD and CB126.

The MC-type inducers of CYP are the so-called coplanar PCBs because the rings can attain a planar configuration at physiological temperatures. These congeners are substituted with chlorine in both para and at least two meta positions and not

substituted at the ortho positions. The chlorines are isosteric to the infamous and most toxic chlorinated xenobiotic - 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, popularly known as dioxin) (Figure 1.2). The resemblance of these types of PCBs to TCDD results in a similar pattern of toxic effects. Removal of any one of these substituents or the addition of one or more ortho-chlorine groups will result in significant loss of MC-type activity (25). In general, these types of inducers have more pronounced acute toxicity than their PB-type inducer counterparts. As MC-type inducers, these congeners increase gene expression of the CYP1A isozyme.

Induction of CYP1A gene expression by MC-type inducers has been extensively studied (Figure 1.3). The initial step of induction is mediated through binding of PCBs to the aryl hydrocarbon receptor (AhR). There are two types of evidence that implicate AhR in induction of CYP1A transcription (36). The first is biochemical in that, within groups of structurally related compounds, there is a correlation between receptor binding affinity and potency as an inducer. The second is genetic, where different inbred mouse strains can differ quantitatively in their responsiveness to aromatic hydrocarbons. The AhR has been studied extensively (37-41).

Prior to binding with a ligand, inactive AhR resides in the cytoplasm of target cell as a soluble complex with a heat shock protein, Hsp90 (40). It has been hypothesized that the Hsp90-receptor interaction stabilizes the unliganded receptor in a configuration that facilitates ligand binding and/or inhibits the unliganded receptor from binding to DNA (36). Once the inducer binds with the AhR, the Hsp90 is released. The inducer-AhR complex does not seem to bind strongly to DNA,

therefore, to facilitate induction, it must dimerize with another protein, known as the aromatic nuclear translocator (ARNT). The ARNT is so named for its assumed role of translocating the ligand-AhR complex from the cytoplasm to the nucleus. It has been shown that ARNT deficient cells will show nuclear accumulation of ligand-AhR complex with exposure to the appropriate inducer ligand. This suggests the primary role of ARNT is not translocation but rather dimerization with the AhR, facilitating DNA-binding capability (36).

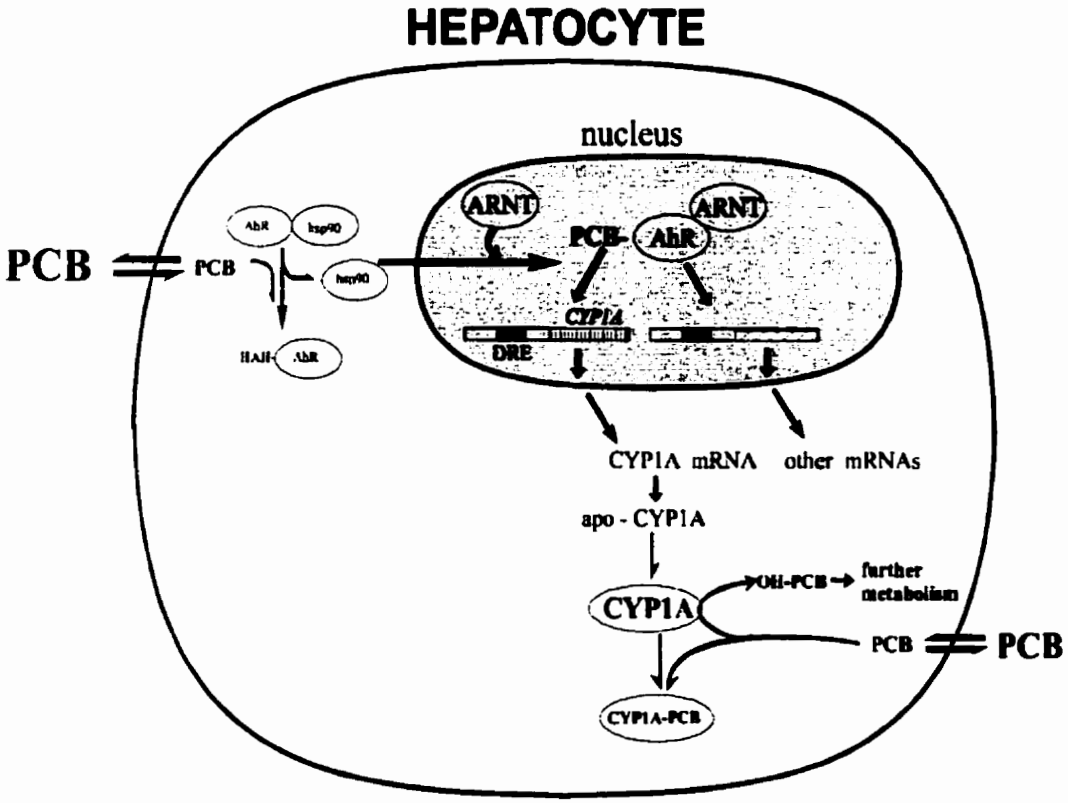


Figure 1.3 - Schematic diagram of a hepatocyte showing the induction mechanism of CYP1A enzymes.

Once in the nucleus, interaction of the AhR complex with upstream specific DNA enhancer sequences, known as dioxin-responsive elements (DREs), induces a

change in the chromatin structure. Chromatin is the mass of dispersed genetic material formed of DNA and protein. This change leads to nucleosome disruption, which allows increased promoter accessibility (42). The CYP1A promoter region is now accessible to its cognate binding proteins and transcription is initiated. With increased transcription, there is an increased production of cytochrome P4501A, which selectively initiates the metabolism of non- and mono-ortho, chlorine substituted PCBs possessing ortho-meta carbons substituted with hydrogens (43).

Mixed inducers are inducers that can activate many enzymes at once. An example, Aroclor 1254, is used as a mixed inducer since it contains PCB congeners, which structurally fit both MC and PB types of enzyme activity.

1.3. Mechanism of CYP hydroxylation of PCBs

The metabolism of PCBs has been well studied and many rules for bioaccumulation and structure activity relationship have been defined (24, 43). Even so, the exact mechanism of the first step in metabolism (hydroxylation) is still unclear. The accepted mechanism of CYP oxidation is based on studies of one of the three crystallized forms of CYPs, P450cam, an enzyme responsible for the stereospecific hydroxylation of camphor.

The reactive site of the CYP enzymes consist of an iron proto-porphyrin IX with a cysteinate as the fifth ligand which leaves the sixth axial coordination site open to bind with molecular oxygen (44) (Figure 1.4). Proton NMR studies of P450cam show the sixth axial site is initially occupied by a water molecule that is displaced upon substrate binding (45).

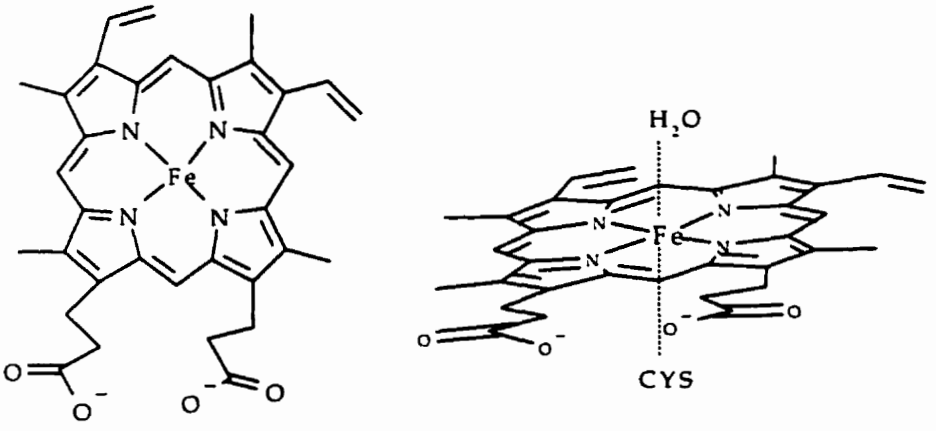


Figure 1.4 - Top and side view of the iron protoporphyrin IX reaction site of cytochrome P450 enzymes. Upon substrate binding, the H_2O ligand is displaced and the site is open to bind with molecular oxygen (CYS = cysteinate).

Hydroxylation of PCBs begins with binding of substrate to the CYP enzyme, which possesses a low spin, ferric iron (Fe^{3+}). This causes the heme iron to change to high spin ferric form (45). The second step involves reduction of iron to the ferrous (Fe^{2+}) state by addition of an electron from a donor molecule, a flavin containing enzyme called NADPH-cytochrome P450 reductase (20). The reduction of the iron now allows it to bind to molecular oxygen to form a dioxygen complex (44). The addition of a second electron results in a peroxoiron(III) complex. Protonation and cleavage of O-O bond brings about the reactive iron-oxo $(FeO)^{3+}$ complex that can directly oxidize the substrate (20, 44). The biotransformed substrate is then released, permitting the CYP enzyme catalyst to repeat the cycle. It should be noted that the rate determining step, namely the first electron transfer, has been observed

spectroscopically while the second electron transfer and oxidation of the substrate occur too rapidly and have not yet been observed.

Aromatic oxidation, in this case PCB oxidation, involves the introduction of a hydroxyl group into the aromatic ring. It is thought that two possible mechanisms exist for the insertion of oxygen onto the aromatic ring. The first involves epoxidation, which is followed by epoxide ring opening. The presence of an epoxide ring intermediate allows for the possibility of intramolecular migration or shift of the group being displaced by the hydroxyl to an adjacent carbocation newly formed on the aromatic ring. With tautomerization of the ketone product, either substituent on the sp^3 carbon can be lost (46). The hydroxylation-induced intramolecular 1,2 shift has been given the name National Institute of Health shift or "NIH Shift" (47) (see Figure 1.5). Studies of the metabolism of individual PCBs have shown that several isomers of hydroxylated metabolites can be formed for each individual congener through the NIH shift mechanism (48, 49).

The second mechanism was developed from evidence where it was shown that there was a quantitative loss of the hydrogen on the hydroxylated carbon and a small deuterium kinetic isotope effect was sometimes observed for hydroxylations meta to a halogen substituent on chlorinated benzenes (50). This could support the theory of direct oxygen insertion into the C-H bond or it could also result from the oxidation of the aromatic ring by a non-concerted mechanism that does not pass through a discrete epoxide metabolite (46).

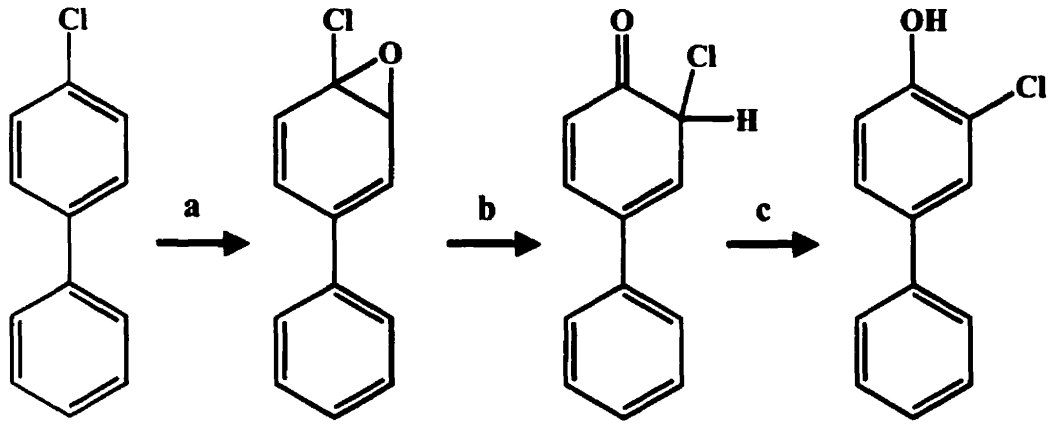


Figure 1.5 - "NIH like" shift involving the hydroxylation of a chlorobiphenyl. Step a - involves epoxide formation followed by a rapid ring opening; Step b - 3,4 shift of chlorine, and the tautomerization of the ketone; Step c - with loss of either the hydrogen or chlorine.

1.4. Metabolite formation during the oxidation of CB153

1.4.1. Nomenclature for OH-PCB metabolites

The chosen nomenclature for numbering hydroxylated PCBs or their derivatized analogues, methoxylated PCBs, is slightly different from general IUPAC guidelines. The OH- or MeO- groups are not given numbering priority. Rather, the chlorine pattern on the biphenyl rings determines the congener number according to the IUPAC PCB numbering rules (1) with corrections by Guitart *et al.* (2), and the OH- or MeO- groups are numbered thereafter. This facilitates direct structural comparison with PCBs because the IUPAC numbering system is so well established and familiar.

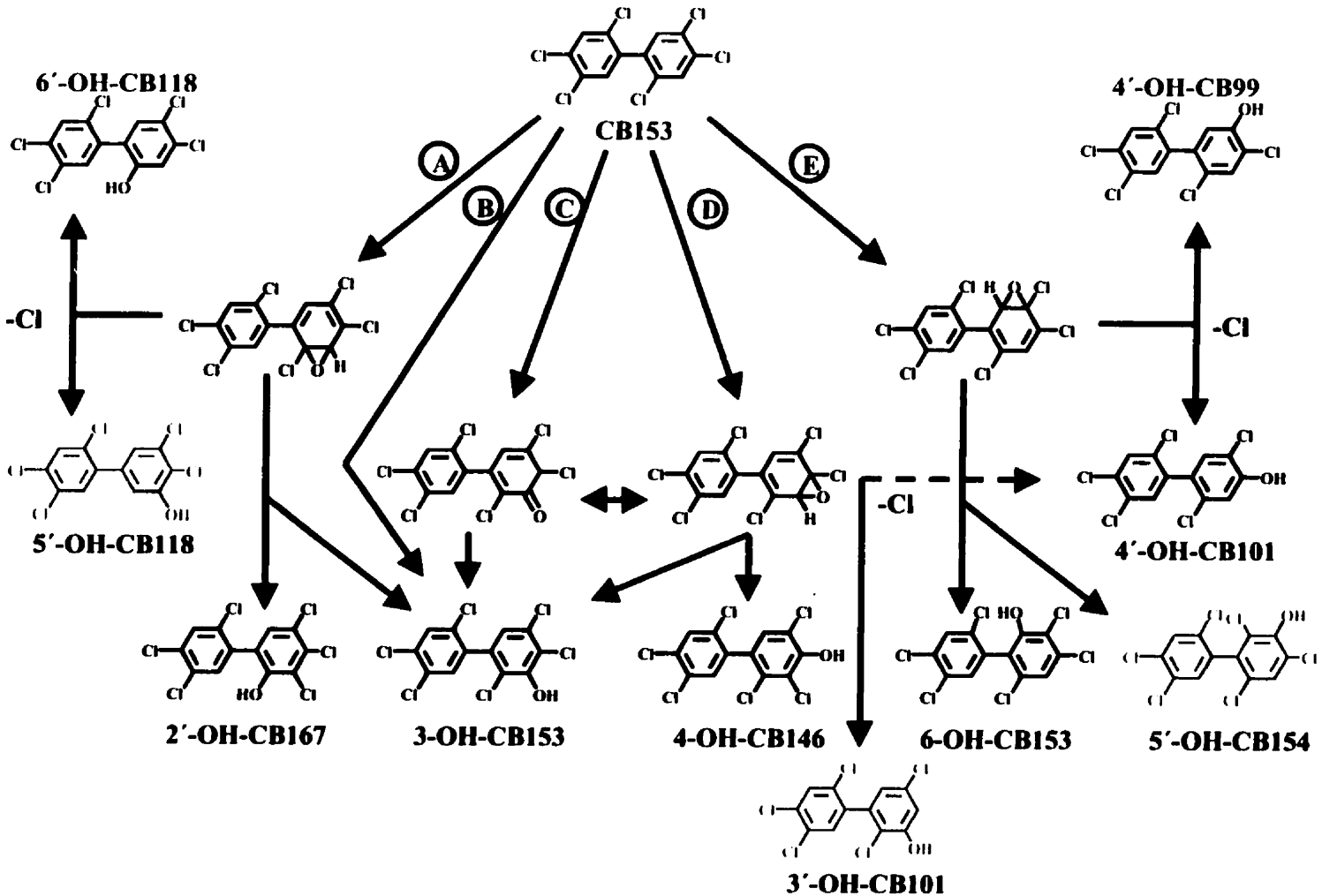


Figure 1.6 - The hydroxylation mechanisms proposed for the cytochrome P450 oxidation of CB153. Shaded structures have not been determined experimentally.

Figure 1.6 demonstrates the numerous metabolites that can form from a major individual PCB congener, such as CB153. It is important to note that though most of these metabolic processes have been demonstrated, the rate of metabolism of CB153 is very slow (51). Gray structures are strictly theoretical and have not been demonstrated in metabolism studies.

Mechanism A involves a 2,3-arene-oxide intermediate that can form one of three possible metabolites. The epoxide intermediate can result in two dechlorination products 5′-OH-CB118 or 6′-OH-CB118 where chlorine is selectively lost. The former metabolite has never been reported while the latter metabolite was formed and excreted by guinea pigs (52), dogs (53) and by *in vitro* incubation with human CYP2B6 microsomes (54).

The epoxide could also rearrange via an NIH shift to form 2′-OH-CB167. This metabolite has been found in both guinea pig (52) and dog (55). The last possible metabolite that 2,3-arene oxide could form involves an NIH shift to form 3-OH-CB153. This metabolite is the most commonly observed metabolite in feeding studies and in *in vitro* work (54). The metabolite, 3-OH-CB153, has been found in feeding studies using rabbits (56), mice (57), rats (56), guinea pigs (52), and dogs (53).

Mechanism B demonstrates the formation of 3-OH-CB153 from direct insertion of a hydroxyl group into the meta position. Glutathione is known to react with arene oxides. When glutathione is added to a microsomal incubation, it saturates the formation of arene oxides, thereby inhibiting formation of metabolites formed

through this pathway. Using this method, Ariyoshi *et al.* demonstrated that dogs can use the direct insertion mechanism to form 3-OH-CB153 (53).

Mechanism C shows the proposed rearrangement of a keto-enol intermediate to form 3-OH-CB153. The keto-enol can also result from the rearrangement of 3,4-epoxide formation generated by Mechanism D. The 3,4 epoxide can under go a 3,4 shift of chlorine to form 4-OH-CB146 or can form 3-OH-CB153. It is also possible to form two dechlorinated metabolites – 4′-OH-CB101 and 3′-OH-CB101, with only the former having been identified previously in guinea pig, rat and rabbit (52, 56, 57).

The last mechanism, mechanism E, involves a 5,6-arene-oxide intermediate, which can dechlorinate to 4′-OH-CB99 or 4′-OH-CB101. Both metabolites have been demonstrated in rabbit (57), rat (56), and guinea pig (52). A 5,6 shift of chlorine would result in 6-OH-CB153 and no shift in chlorine would result in 5′-OH-CB154. The former metabolite, 6-OH-CB153 has been seen in rat studies (56) while the latter metabolite has never been seen experimentally.

Of the many possible metabolites formed from CB153, only two have been identified in blood – 4-OH-CB146 and 3-OH-CB153 (5). These metabolites have been shown to accumulate in plasma and are likely bound to the thyroid hormone transport protein, TTR (58). It would be presumptuous to say that these two metabolites are present in blood from the metabolism of CB153. Two other environmentally relevant PCB congeners can also form these metabolites through these same hydroxylation mechanisms (Figure 1.7) (6). CB146 forms both 4-OH-CB146 and 3-OH-CB153 through direct insert or NIH shift mechanisms while CB138

can form 4-OH-CB146 through an NIH shift. Thus, metabolites to parent PCB relationships are difficult to determine.

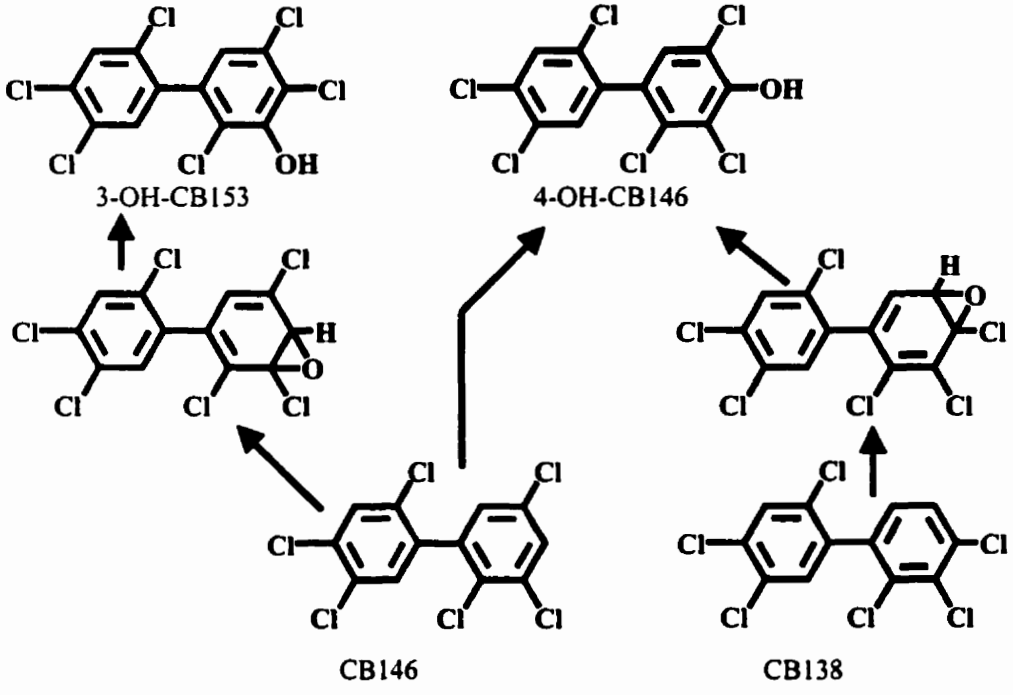


Figure 1.7 - Metabolism of CB146 and CB138 may also lead to the same metabolites as CB153 - 3-OH-CB153 and 4-OH-CB146 (6).

As demonstrated, the metabolism of PCB congeners is complex and suggests that CYP isozymes metabolize their particular PCB substrate by a common mechanism. Despite the number of studies done to date, the exact mechanism of hydroxylation remains elusive. The problem stems from inconsistent methods used in metabolism studies, different number of possible species, isozymes and substrates to investigate, and an inability to get exact X-ray structures of the membrane bound isozymes of CYP. There are numerous reviews on the metabolism of PCBs that can be read for further information (28, 59, 60).

Most species are exposed to a variety of exogenous chemical inducers. The portfolio of anthropogenic compounds to which different organism are exposed is dependent on their particular feeding ecology and environment. Evaluating the mechanism of hydroxylation by one CYP enzyme is therefore complicated by the presence of multiple CYP isoforms in a given wild animal species.

1.5. Other chlorinated hydrocarbon contaminants (CHCs) determined in plasma

Many other contaminants are found at quite high concentrations in plasma and blood. Since most methodology incorporates analysis of other CHCs with PCB analysis, these compounds were also monitored in this thesis. These compounds can be important contributors to environmental contaminant effects of exposed species so they are quickly summarized below.

1.5.1. Bis-2,2-(4-chlorophenyl)-1,1,1-trichloroethane (DDT) and its metabolites

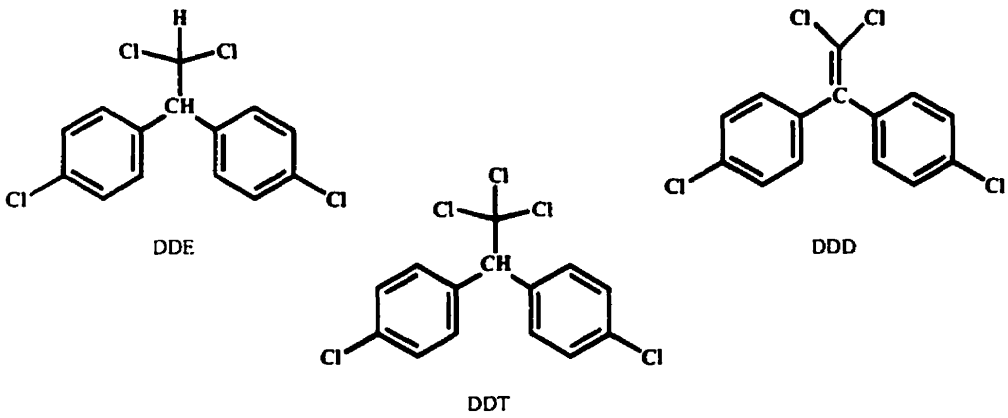


Figure 1.8 - DDT and its metabolites - DDE and DDD.

DDT was introduced in 1945 as an insecticide and technical products consist of both para-para and ortho-para isomers and their dechlorinated analogs. DDT has been banned in most nations, including Canada, the U.S. and Europe but continues to be manufactured and used in the equatorial regions, such as Asia, Africa, Central America and South America. DDT is metabolized to DDE and DDD by the CYP enzymes and both are found in biota (Figure 1.8). DDT and its metabolites are widely dispersed throughout the global ecosystem (61).

1.5.2. Chlordanes

Chlordane, sold as a technical mixture, is a mixture of over 147 compounds (62) of which many minor constituents remain unidentified. Some of the main identified constituents of the mixture include *cis*- and *trans*-chlordane (19% and 24 %), *cis*- and *trans*-nonachlor (7% *trans*) and heptachlor (7%) (Figure 1.9). Some

components, such as heptachlor were synthesized and sold individually. Oxidative products of chlordanes, such as heptachlorepoxide and oxychlordane, have also been found in biota (63) and have been shown to accumulate even more readily than their precursors (64). Technical chlordane was mainly used in non-agricultural applications but was also used to protect crops such as corn and potato (65). Many components in technical chlordane are enantiomeric so research on the enantioselective accumulation/metabolism is currently of high interest (66, 67). Chlordanes are persistent in the environment and readily volatilize so they are globally distributed by long range atmospheric transport.

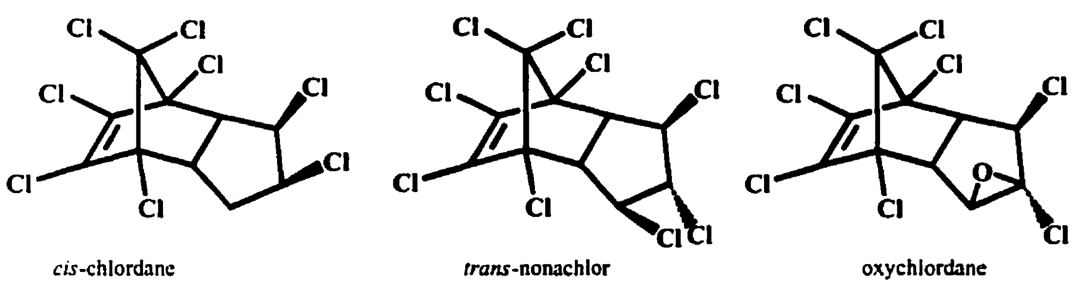


Figure 1.9 - Examples of some of the main chlordane compounds commonly determined in biota.

1.5.3. Hexachlorocyclohexanes (HCHs)

HCHs are used as a technical mixture in agricultural insecticides. Technical HCH mixtures contain three main isomers - α-HCH (55-70%), β-HCH (5-14%), γ-HCH (10-18%) and δ-HCH (impurity) (Figure 1.10). The γ-HCH isomer, also known as lindane, is still used in its pure form and is the biologically active component in technical mixtures. Technical HCH is currently used in China as an insecticide on

seeds, fruit, vegetables and lumber (68). The HCH isomers are less bioaccumulative than other organochlorines since they have relatively low lipophilicity and short half-lives. HCHs are prone to global atmospheric transport because of their volatility (69). α-HCH is enantiomeric and has received increased attention because of rising interest in enantiomeric compounds and their degradation in the environment.

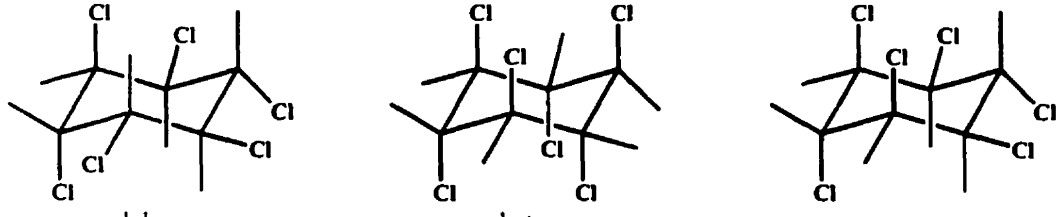


Figure 1.10 - Structures of the three main HCH congeners determined in biota.

1.5.4. Chlorinated benzenes

Hexachlorobenzene (HCB) (Figure 1.11) is the main chlorinated benzene found in biota. HCB is formed as a by-product in the production of lower chlorinated benzenes and pesticides. HCB itself was also used as a fungicide. HCB and the other chlorinated benzenes are persistent, relatively lipophilic and semi-volatile allowing distribution via atmospheric transport. In rats, HCB has been shown to be metabolized to pentachlorophenol (70), indicating HCB as a possible source of pentachlorophenol in some organisms.

1.5.5. Chlorinated phenols

Pentachlorophenol (PCP) (Figure 1.11) is a fungicide that has been used extensively for wood preservation in the lumber industry. PCP has also been used as

an insecticide in the form of sodium salt. PCP products also contain contaminant residues of lower chlorinated phenols and trace levels of dibenzo-*p*-dioxins, dibenzo-*p*-furans, and phenoxy-phenols. PCP is persistent in water and has limited potential for volatilization, thus remaining near local sources. PCP does not bioaccumulate and is rapidly excreted from dosed rats (71). The most important storage compartment for PCP is plasma. This is likely due to its potential to bind with relatively high affinity to the thyroid hormone transport protein, transthyretin (72). PCP has a tendency to remain in plasma and is rarely determined in routine environmental monitoring. This may be because most monitoring involves adipose analysis or because PCP is seldom determined. Pentachlorophenol has been determined extensively as pentachloroanisole and is one of the major pesticides found in arctic air (73).

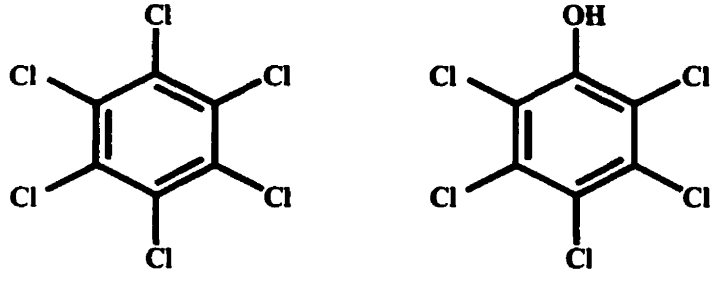


Figure 1.11 - Chemical structures of hexachlorobenzene (HCB) and its metabolite - pentachlorophenol (PCP).

1.6. Basic biochemistry of affected systems

Environmental contaminants with different structures and physicochemical properties can have many common effects that pose a threat to wildlife or humans. Two of the most well studied toxicological endpoints of environmental contamination

by persistent organic pollutants are effects on the thyroid system and on retinol homeostasis. The basic biochemistry of these systems is summarized below.

1.6.1. Vitamin A

Retinoids are the generic term for compounds that include naturally produced compounds, such as retinol, as well as synthetic compounds that show vitamin A type activity. Vitamin A (retinol) is an essential nutrient that plays a key role in embryo development (74), and is involved in the maintenance of differentiated epithelia and mucus secretion (75). Retinoids are stored mainly in the liver but can also be stored in the kidneys, depot fat, lungs and adrenal glands (76). Dietary retinol is ingested as retinyl esters or β-carotene. β-Carotene is cleaved primarily in the intestinal mucosa by an enzyme designated as β-carotene 15,15′-dioxygenase. The resulting products are two molecules of retinaldehyde which are subsequently reduced to retinol by retinaldehyde reductase (77). The dietary retinyl esters are also converted to retinol by hydrolysis in the intestinal lumen. The retinol is then absorbed in the mucosal cell and re-esterified with long chain fatty acids. It is incorporated into chylomicra and transported to the liver via the lymph. Once in the liver, retinyl esters are efficiently transferred to the hepatic stellate cells for storage. About 50-80% of the total body vitamin A is normally stored in the liver stellate cells (also known as Ito cells) as retinyl esters (examples - retinyl palmitate, retinyl stearate). The retinyl esters are then hydrolyzed to retinol prior to retinol mobilization by retinyl palmitate hydrolase enzyme. The mobilization of retinol is a highly regulated process and also involves

the synthesis and co-secretion of a transport protein by either the stellate cells or the parenchymal cells.

Retinol has low solubility in aqueous media and is thus solubilized and transported in blood to peripheral target tissues by a specific transport protein, retinol binding protein (RBP) (78-80). RBP is a relatively small protein composed of a single polypeptide chain of 21 kDa and having one binding site for retinol. The RBP and RBP-ligand complex have been well characterized by X-ray crystallography techniques (81-84). RBP, a highly conserved protein among taxa, is synthesized in hepatocytes and is controlled by the nutritional vitamin A status of the animal (85). RBP is generally complexed with retinol prior to secretion from the cell. RBP is found at average concentrations of about 40-50 μg/ml in human blood.

In circulation, the RBP-retinol complex is associated with another protein known as transthyretin (TTR) (Figure 1.12). TTR is found at higher concentrations than RBP in human blood (200-300 μg/ml) (79) and forms a 1:1 dimer with RBP. This dimer formation is thought to stabilize the interaction between retinol and RBP (86, 87) and prevent glomerular filtration of the small RBP protein (85). It has also been suggested that TTR and RBP are co-secreted by the hepatocytes since TTR is also synthesized in the parenchymal cells (84). The binding sites between TTR and RBP have recently been defined (88) and the TTR:RBP:retinol complex has been crystallized and analyzed by X-ray diffraction analysis (89).

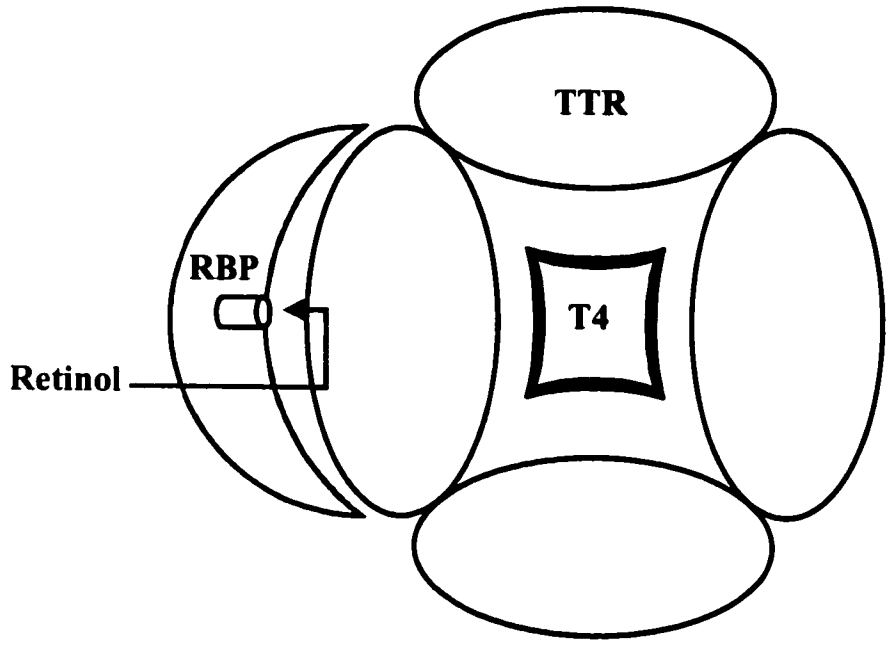


Figure 1.12 - Schematic of the dimer complex formed between retinol binding protein (RBP) and transthyretin (TTR).

Retinol has been suggested to be a good indicator of environmental exposure to xenobiotics (90). In laboratory animals and many wildlife species, PCBs and related compounds have been shown to alter retinoid homeostasis (91). Early investigations on PCBs showed that liver stores of retinol were decreased upon exposure (92-94). This has also been demonstrated in environmentally exposed seals (95). It is thought that PCB induced enzymes are the main cause of the liver depletion in retinol levels (96, 97). Later studies implied that plasma retinol concentrations were also affected by PCB exposure (96, 98-100). This was confirmed in seals exposed to contaminated fish and environmentally exposed Baltic seals (101). The mechanism of this decrease in plasma retinol involves the decreased transport of retinol by RBP (100) and was further complicated by the fact that PCB metabolites may be involved (102, 103).

Thus, retinoids (including retinol) are commonly used as indicators of PCB or CHC exposure (104). CHCs accelerate the metabolism of vitamin A compounds at vitamin A storage and target sites (97). The depletion of vitamin A at target sites signals hepatic vitamin A mobilization and increased retinol transport and consequent liver depletion in vitamin A stores (97). Thus, plasma retinol concentrations may be related to PCB exposure (105) but may be more directly affected by the hydroxylated metabolites of PCBs which have shown to bind to TTR and disrupt the TTR:RBP complex (106).

1.6.2. TTR and thyroid hormone transport proteins.

TTR is one of three main thyroid hormone transport proteins. The other transport proteins are thyroxine binding globulin (TBG) and albumin. Thyroid hormones and their transport proteins have been reviewed extensively (107-111) and will be discussed briefly. The majority of the circulating thyroid hormones in mammals are non-covalently bound to these specific transport proteins (109). The proteins function as a circulating reservoir and act as a buffer against sudden changes in thyroid hormone levels.

TTR (formerly known as prealbumin) is a symmetrical tetramer, consisting of four identical subunits. The amino acid sequence for human TTR has been characterized (112) giving it a molecular weight of 55 kDa and its three-dimensional structure has also been resolved (113). TTR is also a well-conserved plasma protein and is found in most mammalian species and birds. It is found at very low concentrations in reptiles and is absent in fish and amphibians (114-116). TTR is

degraded predominantly in the liver and is also degraded in muscle and skin (117). It has a high production rate and disappearance rate compare to other transport proteins resulting in a half-life of 1-2 days in plasma (118). TTR is the primary thyroid hormone carrier in rodents but is a secondary carrier to TBG in humans (118).

TBG consists of a single polypeptide chain and has a molecular weight of 54 kDa (109). TBG is a high affinity, low-capacity thyroid hormone transport protein and is found at relatively low concentrations in humans (15 μg/ml). Despite its low concentration, it carries the bulk of the thyroid hormones in humans because of its high affinity for the thyroid hormones. TBG is only found in higher mammals and can be totally absent in some humans due to genetic polymorphism (107). This may be evidence that TBG does not play an essential role in thyroid hormone action as these humans have normal thyroid function.

The last major transport protein is the low affinity, high capacity albumin. Human serum albumin has a molecular weight of 66 kDa and has one relatively strong binding site for thyroid hormones and at least five weaker sites.

1.6.3. Thyroid Hormones

Thyroid hormones (iodothyronines) are important in normal growth, differentiation and development. Development of the central nervous system and skeleton are very dependent on thyroid hormones (119). Thyroid hormones regulate metabolic processes such as tissue oxygen consumption, calorigenesis, mineral balance and the synthesis and metabolism of proteins, carbohydrates, and lipids. Because thyroid hormones increase cholesterol biosynthesis and turnover, an increase

in thyroid hormones can result in decreased stores of most lipids and decreased plasma lipid concentration (119).

The production of thyroid hormones is controlled by a negative feedback loop involving the hypothalamus, pituitary and thyroid glands (HPT axis). The hypothalamus produces thyrotropin releasing hormone (TRH, L-pyroglutamyl-L-histidyl-L-proline amide) which causes the anterior pituitary to produce and release thyroid stimulating hormone (TSH, thyrotropin) in blood. TSH binds to receptors in the thyroid gland. The thyroid gland then triggers synthesis and release of thyroid hormones. The system is controlled by a negative-feedback system where pituitary cells are desensitized to TRH resulting in less TSH production and consequently less thyroid hormone production. Two conditions can occur with an excessive or deficient activity of the thyroid gland. Hyperthyroidism is the condition when the thyroid gland is over active and results in an enlargement of the thyroid gland, a rapid heart rate and higher blood pressure. Hypothyroidism can also lead to an enlargement of the thyroid gland but as a result of deficient activity. Other consequences of hypothyroidism include lowered metabolic rate, general loss of vigor, weight gain and cretinism in infants.

Thyroid hormones are produced in the follicular cells of the thyroid gland. Mammals produce mainly 3,3′,5,5′-tetraiodo-L-thyronine (thyroxine, T4) which is considered a prohormone because it has relatively little biological activity (Figure 1.13). Less than 20% of thyroid hormone production in mammals is the 3,3′,5-triiodo-L-thyronine (T3), the physiologically active hormone (Figure 1.13). Most of the T3 found in circulation is generated by mono-deiodination of T4 in the liver and kidney.

There is also local conversion of T4 to T3 in the extrathyroidal tissues such as brain, pituitary and brown adipose tissue. Each thyroid hormone differs in affinity for the transport proteins resulting in differing distribution among the proteins. In humans, T4 binds 68% to TBG, 11 % to TTR and 20% to albumin (120). For T3, the distribution is 80%, 9%, and 11%, respectively (118).

TTR has two available binding sites but normally, only the first site is occupied. The binding affinity of T4 for the main binding site is $7.0x10^{7}$ M^{-1}, which is lower than the binding affinity for TBG $1.0x10^{10}$ M^{-1}. This results in TTR carrying 10-15% of the T4 in blood and an estimation that only 1 in 300 human TTR molecules contains T4 (118). It should be noted that even though the retinol to RBP complex is enhanced by dimerization with TTR, there is no effect of the retinol-RBP complex on T4-TTR interaction. Thus, T4-TTR binding is independent of RBP concentration.

TTR is the only thyroid hormone transport protein that is synthesized in the brain by the choroid plexus (121, 122). TTR was thought to be the only mechanism for transport of thyroid hormones to the brain (123). It was later determined that although TTR null mice have much lower brain T4 levels, TTR was not essential for T4 to reach the brain (124). Schreiber *et al.* (122) and Nilsson *et al.* (125) have reviewed thyroid hormone delivery through brain TTR.

1.6.4. Thyroid Hormone Metabolism

1.6.4.1. Primary Thyroid Hormone Metabolism

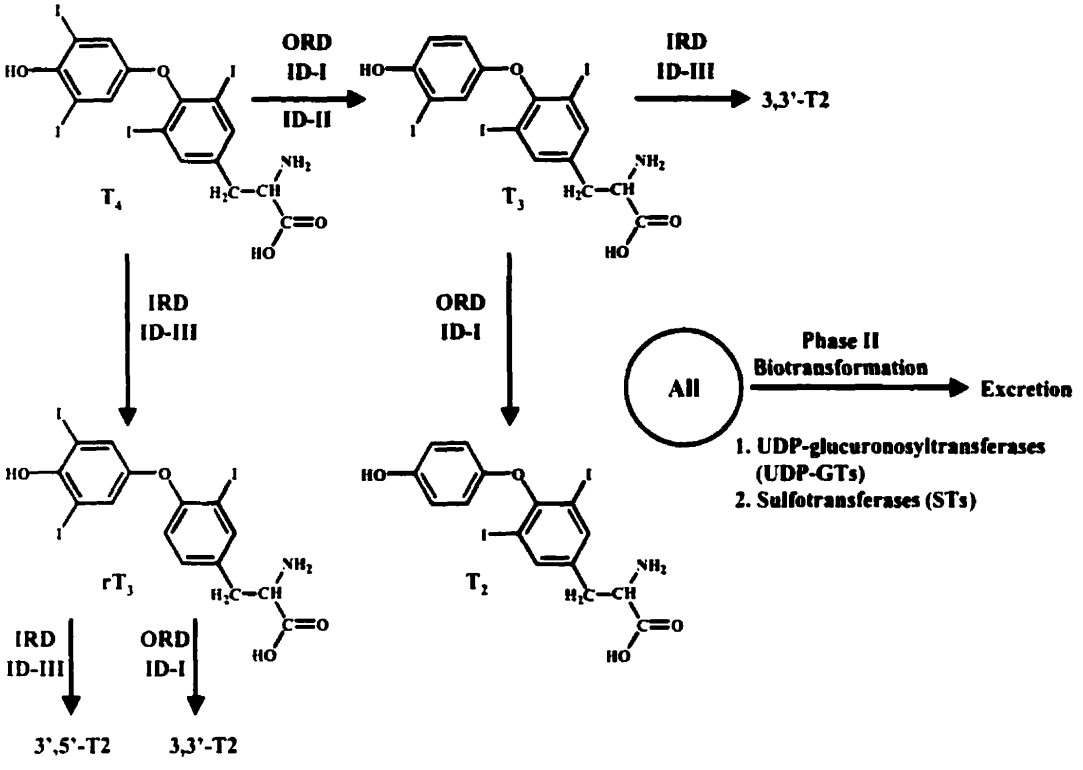


Figure 1.13 - The metabolic deiodination reactions of the thyroid hormones by the deiodinase enzymes.

The majority of circulating thyroid hormones is the prohormone T4. Therefore, there exists an enzyme family distributed throughout the tissues to convert T4 to T3. This family of enzymes is regulated independently to ensure local hormone action. There are three main monodeiodinase enzymes responsible for the cellular Phase I activation and deactivation of the thyroid hormones (126). The enzymes are a family of newly discovered, predominantly membrane bound, selenocysteine-

containing proteins and are located in the tissue microsomal fraction. Figure 1.13 shows a schematic for the deiodination reactions of thyroid hormones.

Type I-5′-deiodinase (ID-I) is the main deiodinase enzyme and also the best characterized. ID-I removes the iodide from mainly the 5′-position (or equivalent 3′ of the hydroxyl ring), in a process called outer ring deiodination (ORD). ID-I is also capable of inner ring deiodination (IRD) of other iodothyronine substrates (127). ID-I is found in the liver, kidney, thyroid and euthyroid pituitary and produces most of the circulating T3 (70%) under normal metabolic conditions. It is also an important enzyme in the removal of reverse T3 (rT3), an inactive product of T4 deiodination. ID-I expression is induced by thyroid hormones. Testosterone in rats has also been shown to stimulate hepatic ID-I (128).

Type II-5′-deiodinase (ID-II) catalyzes the conversion of T4 to T3 in a similar fashion as ID-I. Unlike ID-I, elevated T4 or rT3 deactivates ID-II. ID-II is found in pituitary, brown adipose tissue and the central nervous system and is thought to play a role in maintaining local production of T3.

Type III-5-deiodinase (ID-III) is predominantly located in the central nervous system, placenta, and skin. ID-III only deiodinates the inner ring of T4 and thus produces inactive rT3.

Thyroid hormones and their deiodination products are then further metabolized by Phase II conjugation type reactions involving the para-hydroxyl group of the thyroid hormone. As with most phase II conjugation reactions, the product formed is more water soluble and excreted rapidly in the bile (127). Examples of phase II conjugation reactions for thyroid hormones include conjugating with glucuronic acid

or sulfate by UDP-glucuronosyltransterase (UDP-GTs) and sulfotransferase (STs) enzymes. The STs are cytosolic enzymes whereas the UDPGTs are membrane bound enzymes in endoplasmic reticulum.

1.6.4.2. Secondary Thyroid Hormone Metabolism

UDP-GTs are a family of inducible microsomal enzymes that can differ in substrate specificity. Glucuronidation pathways were reviewed by Visser (129). Common inducers of these enzymes include phenobarbital (PB), 3-methylcholanthrene (3-MC), pregnenolone-16β-carbonitrile (PCN) and PCBs. The induction of UDP-GTs can cause decreases in serum T4 levels and total T3 levels in rats (130). They can also increase TSH in rats in a dose dependent manner (131). Recent evidence suggests that the increase in serum TSH occurs because of T3 glucuronidation (132). This study also concluded that reductions in serum T4 concentrations in PCB treated rats occurred because of the increase in T4 glucuronidation (132).

In contrast to thyroid hormone glucuronide conjugates, sulfate conjugates produced by the STs are not rapidly excreted in bile (133). Instead, thyroid hormone sulfate conjugates are rapidly degraded by the aforementioned deiodination reactions (127). Chopra *et al.* (134) and Anderson (135) summarize the role of sulfation in thyroid hormone metabolism. Sulfation facilitates the IRD of T4 and T3 while it inhibits the ORD of the T4-sulfate conjugate. This indicates that sulfate conjugation results in the inactivation of thyroid hormones.

1.7. Toxicological aspects of hydroxylated metabolites and other halogenated phenolic compounds

Halogenated aromatic compounds, such as PCBs, PCDDs and chlordanes have been studied extensively for their effects on biological activity. The main focus of current research is the effects of metabolites and related phenolic compounds. The latest research on effects of these types of compounds is summarized below. Brouwer *et al.* (136) and Brucker-Davis (137) supply a recent review of the effects of environmental contaminants on thyroid function.

1.7.1. Thyroid Hormone and Retinol Transport

Binding of xenobiotic compounds to thyroid hormone transport proteins was first noted by Marshall and Tompkins (138). They found that o,p'-DDD competed for TBG binding sites but the binding was concluded to be quite weak.

McKinney *et al.* (139) first hypothesized that TCDD and related compounds were likely candidates to bind with thyroid hormone transport proteins. Such an effect for CHCs was not proven until serum retinol concentration in rats and mice was shown to decrease with CB77 exposure and it was determined that the mechanism was not related to aryl hydrocarbon hydroxylase enzymes, a sign of enzyme induction (98, 99). Extrahepatic retinoid concentrations were also significantly reduced with exposure (140). With further investigation, CB77 was shown to reduce not only circulating retinol concentrations and extrahepatic concentrations but also RBP levels, circulating T4 concentrations, and the free T4 index (a measure of free T4 available in plasma) (102, 103). This strongly indicated disruption of the TTR-RBP complex via

competitive binding to the TTR molecule resulting in thyroid hormone displacement and disturbance of the TTR-RBP complex causing increased glomerular filtration of RBP protein (102). Using radiolabelled CB77, Brouwer and van den Berg determined that it was the polar hydroxylated metabolite of CB77 that was causing disruption in thyroid hormone and retinol concentrations in mice and rats exposed to CB77 (98).

Lans *et al.* (141) confirmed the binding of OH-PCBs to TTR (142), determined that structurally diverse compounds such as OH-PCBs, OH-PCDFs, and OH-PCDDs could all bind with high affinity (142) and defined the binding site of TTR using X-ray crystallographic techniques (141). The relative binding potency of some of the OH-PCBs are listed in Table 1.1. The relative binding potency is a ratio of the binding affinity of each substrate to T4. Although Lans *et al.* (142) mentioned that none of the PCBs tested competitively displaced T4 in their assays, McKinney *et al.* continued to theorize a possible interaction between PCBs and TTR (143). Results recently published indicate that certain PCBs do bind to TTR with quite high affinity (144). Also, other halogenated phenolic compounds, such as PCP, brominated phenols and tetrabromobisphenol-A (TBBP-A) have been shown to act as potent competitors for TTR (72, 145). The relative binding potencies of PCBs and other halogenated phenolic compounds are summarized in Table 1.1.

Table 1.1 - Relative binding affinities of PCBs and metabolites to human transthyretin as compared to T4. PCB relative binding potencies were determined by Chauhan et al. (141).

Compound	Parent Compound	Relative Potency for TTR	Reference	Compound	Relative Potency for TTR
OH-PCBs				**PCBs**	
4-OH-CB14	CB14, CB12	3.9	(146)	CB14	0.6
4'-OH-CB35	CB35, CB37	8.4	(142)	CB35	0.06
4-OH-CB56	CB56, CB66	10.2	(142)	CB38	1.9
4'-OH-CB61	CB61	0.7	(146)	CB39	0.2
4'-OH-CB60	CB69, CB75	1.9	(146)	CB80	7.1
2-OH-CB77	CB77, CB66	0.5	(105)	CB127	8.2
5-OH-CB77	CB77, CB79	2.7 or 3.5	(105, 142)	CB169	1.2
6-OH-CB77	CB77	0.06	(105)	CB28	0.05
4'-OH-CB79	CB79, CB77	2.5	(105)	CB33	0.06
4,4'-diOH-CB80	CB80, CB77, CB79	5.4, 5.6	(142, 146)	CB35	0.25
4'-OH-CB82	CB82, CB85	5.0	(142)	CB46	0.05
4,4'-diOH-CB83	CB83, CB82, CB85, CB90, CB97, CB99	13.6	(142)	CB47	0.05
2'-OH-CB105	CB105, CB85	0.09	(142)	CB48	0.08
5'-OH-CB105	CB105, CB107	4.6	(142)	CB52	0.07
4'-OH-CB106	CB106, CB114	0.4	(146)	CB85	0.51
4-OH-CB107	CB107, CB105	5.9	(142)	CB95	0.51
4'-OH-CB121	CB121, CB119	1.6	(146)	CB99	0.2
4'-OH-CB127	CB126, CB127	8.5	(142)	CB110	2.6
Other Phenolic Compounds[1]				CB111	2.7
PCP	HCB	1.74	(142)	CB138	1.8
PBP	-	7.1	(145)	CB153	0.6
2,4,6-triCl-Phenol	-	0.33	(72)	CB162	2.3
2,4,5-triCl-Phenol	-	0.15	(72)	CB180	0.07
2,4,6-triBr-Phenol	-	1.2	(72)		
2,4-diBr-Phenol	-	0.06	(72)		
4-OH-HpCS	OCS	1.1	(147)		
TBBP-A	-	10.6	(145)		
TCBP-A	-	0.8	(145)		

[1] Abbreviations for compounds in Table 1 – PBP – pentabromophenol, triCl – trichloro, triBr – tribromo, diBr – dibromo, 4-OH-HpCS – 4-hydroxyheptachlorostyrene, TBBP-A – tetrabromobisphenol A, and TCBP-A – tetrachlorobisphenol A.

These compounds can competitively displace the natural ligand, T4, from TTR disrupting the transport of thyroid hormones to target tissues. The effect of a xenobiotic binding to TTR is to cause the carrier protein to distort slightly to accommodate a differently shaped compound. This distortion makes RBP incapable of forming the dimer complex resulting in the increased glomerular filtration and decreasing circulating retinol levels. The structure-activity relationships indicate that binding to the TTR protein involves one or two ortho chlorines, as both complete ortho chlorination and no ortho chlorination showed decreased binding affinities for TTR. Di-meta substitution on one or both rings most closely resembles the diiodophenolic ring of thyroxine and these compounds generally show the highest binding affinities (144). There is a divergence of opinion as to whether a hydroxyl group is necessary for binding (142, 144). McKinney *et al.* (144) found that a chlorine group can replace the para hydroxyl group and still have competitive binding capability (e.g. CB127) and that non-chlorinated para-hydroxy biphenyls showed no binding capability. In any case, para-hydroxyl groups significantly enhance binding affinity of chlorinated biphenyls and other halogenated compounds as exemplified by the very high affinities of some of the hydroxylated PCBs (142). Bromination also increases the affinity for TTR binding site as seen by the over four-fold increase in affinity of PBP as compared to PCP (145).

TTR binding is not limited to PCBs, OH-PCBs and chloro/bromo phenols. Several compounds have been shown to interact with TTR, such as TBBP-A (145), chlorophenoxy acids, nitrophenols, and chlorobenzenes (148). The binding affinity of TBBP-A for TTR is the highest known binding affinity for a xenobiotic. Even though

TTR is likely the main protein interacting with xenobiotic compounds, some compounds are able to bind with other transport proteins such as TBG (138, 142, 146) and albumin. These interactions are generally very low affinity interactions and will not be discussed.

The potential disruption of thyroid hormone homeostasis and retinol through the mechanism of either parent PCB or metabolite binding to TTR has important implications. Total plasma concentrations of thyroid hormones and retinol could be altered. This may be especially important for some species that lack the TBG transport protein and are more dependent on TTR transport to target tissues. TTR is involved in transport of thyroid hormones to the brain through the cerebral spinal fluid-blood barrier (149). This may result in chemicals passing through the barrier to the choroid plexus and affecting brain function or more importantly, brain development. Another scenario involves placental transport of thyroid hormones and retinol. The developing fetus is initially dependent on maternal sources of thyroid hormones and retinol until it can develop its own system and is therefore susceptible to altered thyroid hormone and retinol homeostasis. Subsequent to dependency on maternal sources, the fetus may be exposed to xenobiotic compounds as they pass through the placental barrier bound to maternal transport proteins.

1.7.2. Thyroid Hormone Metabolism

1.7.2.1. Thyroid hormone sulfotransferase

The sulfotransferases (ST) are important enzymes in the metabolism of thyroid hormones and may be important for the control of thyroid hormone concentrations

when ID-I activity is low, such as in the developing fetus (150). Visser *et al.* (133) developed an assay that uses cytosolic isozymes isolated from human and rats liver. They determined PCP and 2,4,6-tribromophenol to be potent inhibitors of the sulfation reaction. Both compounds are found in human plasma (151). Schuur *et al.* (152, 153) determined that the 50% inhibition concentration (IC50) for OH-PCBs, PCP, OH-PCDDs and OH-PCDFs was in the low micro molar to nano molar range which is within or below the residue concentration range of some metabolites in blood. PCP was the most potent inhibitor with an IC50 of 0.005 μM. This study also addressed the structural requirements for ST inhibition. Compounds must have a hydroxyl group and the hydroxyl group can be in the meta or para position. Planarity of the molecule does not seem to play a major role but halogen substitution increased inhibition potency. Schuur et al (154) later determined isozyme specificity for each of the inhibitors. Male and female rat isozymes did not differ in activity. The OH-PCBs tested did not inhibit the sulfotransferase activity using the human SULT1A3 isozyme but inhibited the human SULT1A1 isozyme.

Thus OH-PCBs and other halogenated phenolic compounds can affect thyroid hormone concentrations *in vitro* and at the concentrations commonly determined in plasma. The inhibition seems to be isozyme specific and will vary among individuals because of varying isozyme concentrations.

1.7.2.2. Thyroid hormone UDP-Glucuronyl-transferase (UDPGTs)

Increased biliary excretion of thyroid hormones as glucuronic acid conjugates was one of the first observations of thyroid hormone disruption in rats exposed to

PCBs (155, 156). Exposure to PCB mixtures (130, 131, 155, 157-160), such as Aroclor 1254 and Kanechlor 400 as well as individual PCB congeners, such as CB77 (161), CB169 (162), CB126 and CB156 (163, 164), all increased T4 glucuronidation in rats. The increased glucuronidation generally results in decreased plasma levels of T4 because of the increased clearance. UGTs are suggested to be controlled via the arylhydrocarbon receptor (AhR) transduction pathway (165) and this mechanism is hypothesized to be the dominant mechanism for thyroid hormone reduction through dioxin-like CHCs (166).

1.7.2.3. Thyroid Hormone Deiodinase activity

Adams *et al.* (167) showed that the metabolites of CB77 could inhibit hepatic ID-I activity in rats exposed to CB77. ID-I activity is one of the major activation routes for the formation of the active T3 hormone from the prohormone T4. Rickenbacher *et al.* (168) showed that PCBs could also be involved in the inhibitory mechanism that would result in decreased availability of T3 in the target tissue.

Brain ID-II activity can also be affected by PCB exposure. Morse *et al.* (169) showed that ID-II activity was increased with exposure to CB169 and CB77 in pregnant rats, their fetus and their offspring (169). They concluded that hydroxylated metabolites accumulated in fetal plasma causing fetal T4 plasma levels and fetal brain T4 levels to decrease (170). The resulting local brain hypothyroidism caused an increase in ID-II activity to compensate for the lower T4 concentrations so that brain T3 levels would be maintained (170).

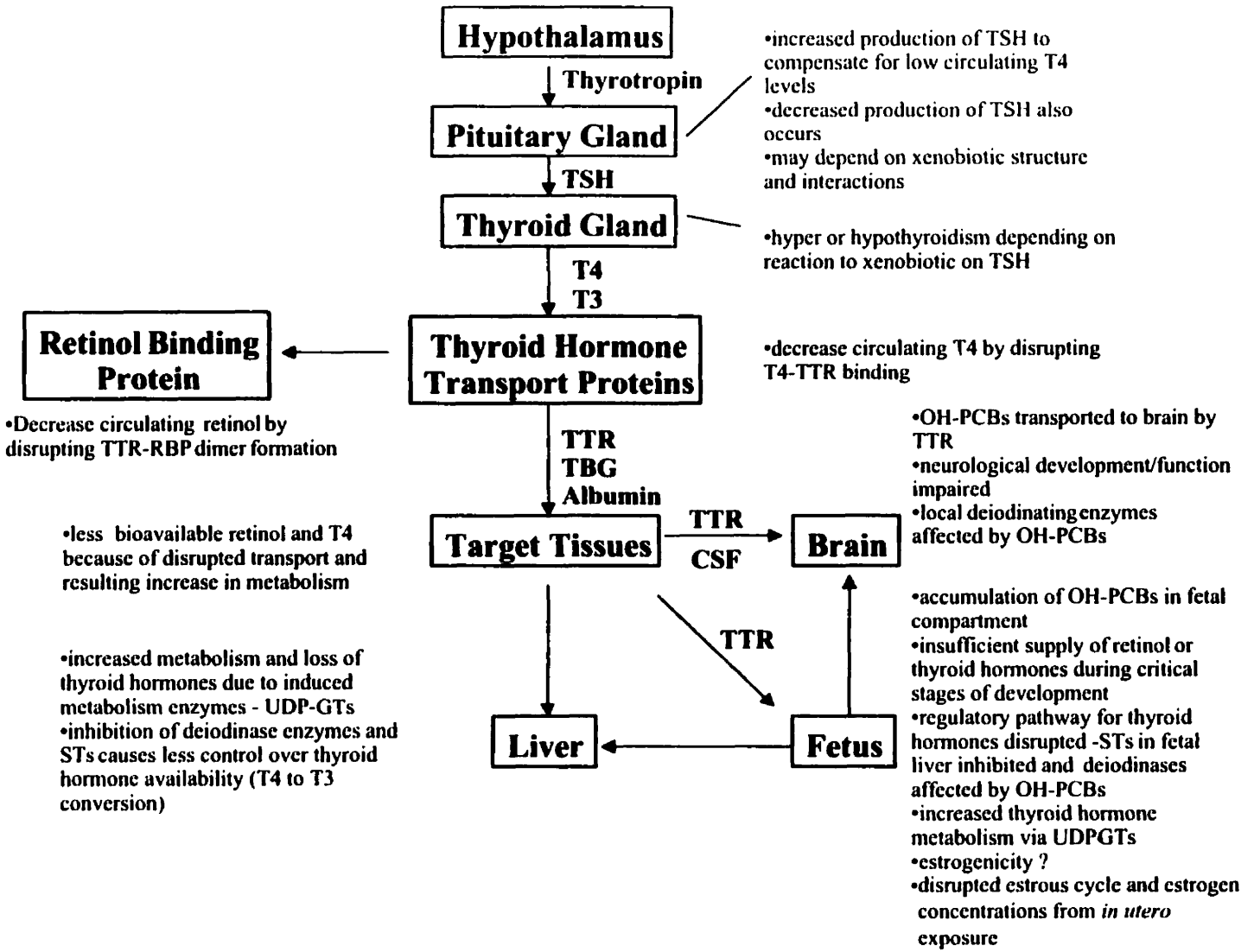


Figure 1.14 - Summary of effects of phenolic compounds and other CHCs on the thyroid hormone system.

A summary of the thyroid related effects of OH-PCBs and other xenobiotics are summarized in Figure 1.14. Although most mechanisms have been determined *in vitro*, many remain to be confirmed *in vivo*. There remains many confounding results that require further explanation.

Many of the effects shown in Figure 1.14 have been recently confirmed in rat dosing studies, especially for transplacental passage and accumulation in the fetus. When pregnant, female mice were intravenously dosed with 4′-OH-CB79, a metabolite of CB77, both fetal and maternal T4 levels decreased in a dose-dependent manner (171). The placental transfer of 4′-OH-CB79 was efficient, resulting in fetal accumulation in liver and plasma. Fetal plasma levels were two to five times those of the mother (55, 171, 172). Fetal plasma from exposed rats was shown to have free T4 and total T4 concentrations that were 64 and 55% as compared to the controls (172). These results have been recently confirmed in a study that involved orally dosed, pregnant rats (173). Rats dosed with 4′-OH-CB109 showed an accumulation of the metabolite in the fetal compartment with a fetal to maternal ratio of 11, 2.6 and 1.2 for the liver, cerebellum and plasma, respectively. At gestational day 20, fetal plasma FT4 and TT4 levels decreased 89 and 41% as compared to controls (173). This is the first study to show fetal accumulation of an OH-PCB in the brain. Accumulation in the brain is likely caused by the transfer of TTR-OH-PCB through the choroid plexus, which then passes through the cerebral spinal fluid to the brain. This mechanism has been shown to be the main transfer of TTR-T4 and supply of T4 to the brain (122). The accumulation in the brain caused forebrain T4 concentrations to decrease by 35% (173) and brain deiodinase to increase (170, 173). This decrease in brain T4 has also

been demonstrated in rats exposed to PCP (174), which is also likely bound to TTR (72).

When *in utero* exposed female rats were followed to maturity, they showed decreased plasma estrogen concentrations and disturbed estrous cycles (increased 82% for high dose group) (175). The effects shown from the *in utero* exposed rats may implicate OH-PCBs as having estrogenic properties. Thus, the effects of OH-PCBs can include both thyroid hormone disruption and possibly sex hormone disruption.

1.8. Other possible toxicological effects of OH-PCBs

1.8.1. Estrogen Receptor Binding

OH-PCB binding to the estrogen receptor binding was first noted by Korach *et al.* (176). They demonstrated that OH-PCBs were able to bind to uterine estrogen receptor protein but with quite low binding affinity. It was noted that increasing ortho chlorination increased the binding activity. None of the congeners tested have been found in plasma but some of them have been determined in excreta of dosed animals (177) and through microbial metabolism (178).

Later, using a luciferase reporter gene as a measure of estrogenicity, Kramer *et al.* (179-181) reported the antiestrogenic activity of 11 OH-PCBs. This study used a stably transfected human breast adenocarcinoma (MCF7) cell line and used both environmentally relevant and non-relevant OH-PCBs. Only two of the OH-PCBs tested, 4,4′-diOH-CB80 and 4′-OH-CB159, showed an estrogen receptor mediated mechanism of action, weakly inhibiting estrogen binding to its receptor. The former

metabolite has been identified in mouse feces (177) while the latter has been shown to accumulate in human (151) and polar bear plasma (Chapter 5). The remaining OH-PCBs tested were considered "anti-estrogenic" as they affected cell viability and did not interfere in binding. Kramer and Giesy (181) then tested the binding affinity using a competitive binding assay of these same congeners to the cytosolic calf uterine estrogen receptor (with an additional 15 OH-PCBs). All but three OH-PCBs showed specific binding capacity to the calf uterine estrogen receptor. A further study involving the MCF-7 human breast cancer cell line showed that some OH-PCBs could significantly increase cell proliferation and were generally more active than the PCBs tested (182).

Another study by Connor *et al.* (183) demonstrated OH-PCBs potential binding to the mouse and rat cytosolic estrogen receptor but with relative binding affinities 700 - 19000 times less than the natural ligand, 17β-estradiol. They also noted several congeners exhibited antiestrogenic activity. Another study using similar OH-PCB congeners and measuring a different estrogen response (luciferase activity in stably transfected HeLa cells or chloramphenicol acetyl transferase reporter gene activity in MCF-7 cells) confirmed that the OH-PCBs had little to no significant activity (184, 185). Investigations into synergistic responses of xenoestrogens using PCBs and OH-PCBs at low concentrations also proved OH-PCBs to be inactive (186). Matthews and Zacharewski (187) tested OH-PCBs for their potential to bind to the estrogen receptors from three different species. They tested both environmentally relevant OH-PCBs and non-relevant OH-PCBs that have been shown previously to bind to the receptors. Only the non-relevant OH-PCBs were competitive for the

estrogen site. The most recent study combined metabolism using liver microsomes with an estrogen-binding assay (188). Again, only the environmentally non-relevant dichlorinated hydroxy biphenyls with the chlorine and hydroxyl groups on different rings showed very low estrogen receptor binding capability.

Thus, the estrogenic potential of OH-PCBs through estrogen receptor binding was inconclusive. Although some congeners were shown to be very weakly active to the estrogen receptor, structure activity concluded that the necessary structure requires an unhindered para-OH on one ring and chlorination on the other (118, 181). These congeners have never been found at detectable circulating concentrations. The tests for estrogenic and anti-estrogenic activity proved to be highly variable and dependent on the chosen cell system and the type of response monitored. Structure activity relationship of estrogen receptor binding of xenobiotics and phytoestrogens was extensively covered by Kuiper *et al.* (189).

1.8.2. Estrogen Metabolism and the sulfotransferases

A recent and more convincing study has implicated OH-PCBs as possibly having indirect estrogenic effects. Kester *et al.* (190) determined that OH-PCBs can affect the metabolism of estrogen since they are potent inhibitors of human estrogen sulfotransferase. These enzymes are similar to the ones mentioned previously for thyroid hormones. They act as a removal mechanism for conjugating estradiol by making it more water soluble to promote excretion. This study found that at physiologically relevant concentrations (ranging from nanomolar to picomolar

concentrations), many OH-PCBs inhibited the estrogen sulfotransferase. This would, in theory, make estradiol more bioavailable at the target tissues. In addition, the most potent inhibitors were the environmentally relevant congeners that are commonly found circulating in plasma (5, 151, 191, 192).

1.8.3. Other possible modes of action of OH-PCBs

OH-PCBs also seem to have effects on mitochondria and other cellular processes. De Haan *et al.* (193) showed that gap-junctional intracellular communication was disrupted by CB77 and its hydroxylated metabolites *in vitro* using hepa1c1c7 cells. Aroclor 1254 was later shown to inhibit gap-junctional intracellular communication with concomitant increase in cell proliferation and decreased apoptosis (194).

Using glutamate/malate as substrate, Nishihara showed that 4′-OH-CB3 was twice as effective as CB3 at inhibiting state-3 respiration of mitochondria (195). Ebner and Braselton showed that para hydroxylated PCBs with adjacent chlorines (i.e. those with an identical structure to compounds retained in plasma) inhibited succinate and valinomycin swelling and oxygen intake in rat hepatic mitochondria (196). They also noted increased efficiency with increasing chlorination with the compounds they tested. These results indicate that OH-PCBs may influence cell function by interfering with mitochondrial functions that affect movement of ions across the inner mitochondrial membrane and cellular respiration. This could cause adverse effects on energy coupling and regulation of metabolism. Lans *et al.* (197) showed that CB77

and its metabolites could act *in vitro* as uncouplers of oxidative phosphorylation in rat liver mitochondria. The metabolites most effective as acting as uncouplers were the non-retained metabolites where the hydroxy group was located on an ortho position. In another study, 4,4′-diOH-CB80 was shown to be the most active inhibitor of state-3 respiration in the presence of succinate (198). These studies indicate that OH-PCBs can significantly affect mouse and rat hepatic mitochondrial oxidative phosphorylation. The effects seem to be substrate specific and dependent on the structure of the OH-PCB. More studies are necessary to determine the structure activity relationships as well as if physiologically relevant concentrations of OH-PCBs are high enough to elicit these effects.

Finally, hydroxylated metabolites are capable of forming reactive quinones that may react with nitrogen or sulfur nucleophiles in cells. This may allow metabolites to bind to cellular macromolecules and cause glutathione depletion, oxidative stress, and possibly cell death (199).

1.9. Current state of knowledge on the analysis OH-PCBs in biota

Interest in OH-PCBs is rising since many of the effects seen in experimental animals and accidental exposures cannot be explained solely through known mechanisms of the parent PCB congeners.

OH-PCBs were first discovered in Baltic seal and Guillemot excreta by Jensen *et al.* in 1975 (200). Since then, in controlled PCB dosing studies, OH-PCBs were determined in the excreta of many animals, in an attempt to assess metabolism rates and mechanisms (58, 59). Their presence in excreta suggested that OH-PCBs have no

toxicological potential since they were being excreted by the organism. Brouwer *et al.* (102, 103) re-ignited hydroxylated metabolite interest when they demonstrated that a metabolite of CB77 (4'-OH-CB79) could disrupt retinol and thyroid hormone levels through binding with TTR in blood as described above.

This led to the first determination of OH-PCBs in blood of a normal exposed human population as well as Baltic seal and Aroclor treated rats (5). Since the first chromatogram of OH-PCBs in biota, there have been few studies that accurately quantitated OH-PCBs in blood. OH-PCBs have been determined in plasma from humans (5, 151, 192, 201, 202), polar bears (203, 204), Baltic Sea seals (5), eagles (205, 206), albatrosses (191) and salmon (207). OH-PCBs have only been quantitated in a limited number of species, which include human (151, 192), polar bear (204), albatross (191) and eagles (205, 206).

Another early study by Zitko *et al.* (208) found OH-PCBs in bird eggs, white shark liver and whole fish homogenate. No standards were available at the time of the experiment therefore only the total number of peaks above detection was identified. This is also true for a study that identified OH-PCBs as contaminants in human milk (209). Only one congener was available for quantitation and concentrations seemed quite low. In a recent study, similar congener patterns of OH-PCBs were determined in liver and adipose tissues of exposed pigs (210). Thus, tissue distribution and the effect of the distribution on the OH-PCB congener patterns requires further research.

Accurate quantitation of OH-PCBs requires proper methodology and analytical standards. Standard synthesis has been described by Bergman *et al.* (211), Safe *et al.* (212) and Bauer *et al.* (213). There have been few publications describing the mass

spectrometry of OH-PCBs and their methylated derivatives. Bergman *et al.* (211) first described the fragmentation of MeO-PCB standard using both electron impact ionization and negative chemical ionization mass spectrometry. They did not determine the effects of temperature and pressure on the fragmentation of MeO-PCBs. There have been limited descriptions of the fragmentation of OH-PCBs and their methylated derivatives by mass spectrometry. These are summarized by Lötjönen (214) and Bergman (211).

There have been few publications on methodology for the quantitation of OH-PCBs. Sandau and Norstrom described the methylation techniques used in the analysis of OH-PCBs (203) and Rozemeijer *et al.* (215) described the extraction and methylation of microsomal extracts. Both groups concluded that the ion pair alkylation (IPA) technique described by Hopper *et al.* (216) was the superior methylation technique for OH-PCBs. Methylation using diazomethane was determined to be as good as IPA for higher chlorinated PCBs (203) and is the main method of derivatization used in most laboratories analyzing OH-PCBs (5). Trimethylsilyl derivatives have also been used in the analysis of OH-PCBs (217, 218) but standard availability may be a problem since most standards are only available as the methoxy derivatives.

Extraction and cleanup of OH-PCBs have been described for the analysis of excreta of seals and Guillemots (200). Extraction from plasma is more difficult as protein binding and coagulation can adversely affect recoveries. A brief description of an extraction method was described by Bergman *et al.* (5) and only recently described in detail by Hovander *et al.* (219).

1.10. Objectives and scope

PCBs have only been weakly associated with many of the toxicological endpoints seen in animal exposure experiments. Many of the effects observed in animals may be attributable to their hydroxylated metabolites. OH-PCBs have specific mechanisms that have been determined *in vitro* but need application at the organism level. Accurate extraction and quantitation of OH-PCBs in tissues and correlation analysis with the biological measures of these specific mechanisms will help determine whether OH-PCBs are able to exert their effects at the organism level. Many of the compounds seen in plasma had not been identified (5), therefore more work was needed for the identification of more OH-PCBs and other halogenated phenolic compounds (201).

To understand the effects of OH-PCBs, more species need to be screened for the presence of these metabolites. This will allow a better understanding of patterns of formation and retention, determination of susceptible population and allow a better evaluation of the toxicological potential if their chemical residues can be related to biological measures.

There was very little information available on OH-PCBs when this thesis began. Thus, methodology for the efficient extraction and quantitation of OH-PCBs was needed for the determination of this class of compounds in plasma. It was quickly determined that OH-PCBs were not the only compounds found in the phenolic compound fraction, therefore the characterization of these other compounds was explored along with the OH-PCBs. More quantitative standards became available

allowing better characterization and more accurate quantitation of the phenolic compound fraction. This thesis describes the methodology that was developed for the extraction and quantitation of OH-PCBs and other halogenated phenolic compounds in whole blood and plasma from a variety of species. The halogenated phenolic compound fraction was further characterized using all the commercially available standards as well as by custom synthesizing particular compounds. Humans and polar bears were then screened for the presence of halogenated phenolic compounds, focusing on OH-PCBs. The chemical residues in human and polar bear were then compared with biochemical measures, such as thyroid hormone and retinol concentrations, to determine possible links between *in vitro* modes of action and levels of OH-PCBs and other halogenated phenolic compounds.

Chapter 2. Derivatization of OH-PCBs and Other Phenolic Compounds and the Optimization of GC-ECNI-MS Conditions in their Identification and Determination in Polar Bear Plasma

2.1. Background

At present, there is limited analytical data available for the analysis of OH-PCBs and other halogenated phenolic compounds (5, 211, 212, 220). No validation exists for the techniques currently used. In this chapter, we address the basic analytical chemistry of the phenolic compound fraction by optimizing their detection.

Most organic environmental contaminant analysis is performed using gas chromatography. Many phenolic compounds have a tendency to elute as long tailing peaks and generally do not chromatograph well. For these types of compounds, derivatization is generally employed to reduce the polarity of the hydroxyl group and improve the chromatographic properties. The most common derivatization technique is methylation. There are currently no studies examining the various methylation reactions available for OH-PCBs. Therefore, three different derivatization techniques were explored to determine which was the most quantitative and free of interfering byproducts. Due to the number of unknown compounds in the phenolic compound fraction (201), gas chromatography coupled with mass spectrometry (GC-MS) was employed so that compounds could be partially characterized by their particular monitoring ions and quantitated using a selected ion monitoring (SIM) program. To maximize sensitivity for full scan analysis of environmental samples, electron capture

negative ionization (ECNI) mass spectrometry with methane reagent gas was chosen as the detection system. The effect of source temperature and pressure on ECNI- mass spectra were studied so that optimal conditions and suitable ions could be selected to maximize response

2.2. Gas Chromatography Conditions

Table 2.1 - GC temperature programs for the different polarity columns used in the identification of OH-PCBs in plasma.

Column	Injector Temperature (°C)	Detector Temperature (°C)	GC Temperature Program
DB-1 & DB-5	250	280	80°C for 2 min. 10°C/min. to 250°C for 5 min. 5°C/min. to 300°C
DB-1701	250	280	80°C for 2 min. 10°C/min. to 200°C for 5 min. 5°C/min. to 280°C for 7 min.
DB-210	250	280	80°C for 2 min. 6°C/min. to 180°C 3°C/min. to 240°C for 8 min.

GC-ECD was performed on a Hewlett-Packard (Atlanta, GA, USA) 5890 instrument equipped with a ^{63}Ni ECD detector and HP7673A automatic injector. The GC was fitted with a fused silica DB-5 column ([5%-phenyl]dimethylpolysiloxane, J&W Scientific Inc., Folsom, CA, USA, 30 m x 0.25 mm i.d., 0.25 μm film thickness). The carrier gas was helium and the ECD makeup gas was 5% methane-95% argon. All injections were 2 μl in volume and made in splitless mode. The injector port and

interface temperatures were 250°C and 280°C, respectively. The GC temperature program was the same as that used in the mass spectral analysis described in Table 2.1.

GC-MS using electron capture negative ionization (ECNI) was performed on a Hewlett Packard (Atlanta, GA, USA) 5890A Series II gas chromatograph equipped with an HP 7673A automatic injector and a Hewlett Packard 5988A mass spectrometer. Helium was used as the carrier gas and head pressure was set at 80 kPa. All injections (2 µl) were made in splitless mode. For OH-PCB peak identification, injections were made on four different columns of varying polarity. All GC columns were 30 m, 0.25 mm i.d. and 0.25 µm film thickness. The GC head pressure was set at 80 kPa with the purge set at 3 ml/min and a final column flow of 1 ml/min. Four columns were used in the analysis: DB-1 (dimethylpolysiloxane), DB-5, DB-1701 ([14%-cyanopropylphenyl]-methylpolysiloxane) and DB-210 ([50%-trifluoropropyl]-methylpolysiloxane), J&W scientific (Folsom, California, USA). For the identification of OH-PCBs, mass spectra were collected at a speed of 0.5 scans per second, scanning from 50 to 550 amu. Three ramping programs were used depending on column and ramping conditions; they are listed in Table 2.1. For routine mass spectral analysis, a DB-5 column was used with the conditions indicated in Table 2.1. Injector temperature was set at 250°C. Mass spectrometry was performed with selected ion monitoring in ECNI mode using methane (99.99% pure) as the reagent gas. The reagent gas source pressure was 2.5 x 10^{-4} torr at the inlet and the source temperature was 140°C.

2.3. Standards and Chemicals

All solvents were residue analysis grade purchased from EM Science (Gibbstown, NJ, USA). Sulfuric acid (Trace metal grade) and Alumina (neutral - Brockman Activity I certified 60-325 mesh) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Basic aluminum oxide was activated by heating to 300°C for 3 hours then deactivating with 0.5% (w/w) doubly distilled and deionized water and stored in a capped (Teflon lined) glass bottle. Concentrated hydrochloric acid (37%) was purchased from BDH laboratory supplies (Poole, England).

The remaining chemicals - trimethylsilyldiazomethane, N,N'-diisopropylethylamine, iodomethane, tetrabutylammonium hydroxide, methylamine, urea, sodium nitrite - were purchased from Aldrich Chemicals (Milwaukee, WI, USA).

Table 2.2 - The four standard mixtures used for identification of OH-PCBs in polar bear plasma. All standards are shown as biphenylols but were analyzed as MeO-derivatives.

Mixture A	Conc. (pg / µl)	Mixture B	Conc. (pg / µl)	Mixture C (^{13}C labeled)	Conc. (pg / µl)	Mixture D	Conc. (pg / µl)
PCP		PCP	1000	PCP	1000	PCP	1000
3-OH-CB85	1000	4'-OH-CB120	1000	4'-OH-CB61	1000	4'-OH-CB120	585
3-OH-CB118	1000	4'-OH-CB107	1000	4'-OH-CB120	1000	4'-OH-CB107	495
4-OH-CB109	1000	4-OH-CB146	1000	4'-OH-CB104	1000	4-OH-CB188	285
3'-OH-CB138	1000	4'-OH-CB130	1000	4-OH-CB187	1000	3-OH-CB153	310
4-OH-CB187	1000	4-OH-CB187	1000	4'-OH-CB159	1000	4-OH-CB146	276
3'-OH-CB180	1000	4'-OH-CB172	1000	4'-OH-CB172	1000	3'-OH-CB138	223
						4'-OH-CB130	130
						3'-OH-CB187	254
						4'-OH-CB175	228
						4-OH-CB187	1000
						4'-OH-CB159	518
						3'-OH-CB180	247
						4'-OH-CB172	316
						4-OH-CB193	390

Standards for the identification of OH-PCBs were obtained from multiple sources. Table 2.2 lists the four different mixtures used in the analysis. Mixtures A to C were made from standards supplied by Wellington Laboratories (Guelph, ON, Canada). Mixture C contained ^{13}C-labeled compounds. Mixture D was donated by Åke Bergman (University of Stockholm, Sweden). At the time of the thesis, these were the only OH-PCBs that were commercially available for identification and quantitation.

Authentic standards of 2,4-dichlorophenol, 2,3,4,6-tetrachlorophenol and PCP were acquired from the EPA Research Triangle Park (Research Triangle Park, NC, USA). Standards of 4′-OH-CB9, 4-OH-CB9, 3′-OH-CB61, 4′-OH-CB61, 2′-OH-CB106, 4′-OH-CB106 were purchased from ULTRA Scientific (North Kingstown, RI, USA). 4′-OH-CB165, 3′-OH-CB85, 4′-OH-CB86, 6-OH-CB86, 4′-OH-CB93, and 6′-OH-CB93 were purchased from Accustandard (New Haven, CT, USA). A performance standard, 4′-Me-4-MeO-CB112, was a custom synthesis by Dr. Bob Wightman (Carleton University, Ottawa, ON, Canada) and was added to samples prior to mass spectral analysis to serve as a correction factor for run to run variability.

Polar bear plasma samples were collected by the late Dr. Malcolm Ramsay (University of Saskatchewan, Saskatoon, SA, Canada). The polar bear plasma pool used in this study consisted of plasma from 6 bears from Churchill, Manitoba, Canada collected in May 1996.

2.4. Derivatization and Derivatization Agents

Since gas chromatography (GC) is still the preferred method of separation of OH-PCBs, and they give poor GC peak shapes, OH-PCBs must be derivatized in order to be efficiently quantitated. Methylation is the most common derivatization and is usually accomplished using diazomethane (5). Diazomethane is both toxic and explosive and must be used circumspectly. Thus, less toxic methylating reagents were considered. To determine the best derivatization technique ion-pair alkylation and derivatization using trimethylsilyldiazomethane were compared to a diazomethane methylation procedure.

2.5. Derivatization Procedures

Because of limited standard availability at the beginning of this research, a mixture of the following six synthetic OH-PCBs – 4′-OH-CB9, 4-OH-CB9, 3′-OH-CB61, 4′-OH-CB61, 2′-OH-CB106 and 4-OH-CB193 were used for testing the three different methods of methylation.

2.5.1. Ion-Pair Alkylation (IPA)

A method described by Hopper (216), originally used for the methylation of pentachlorophenol and chlorophenoxy acid herbicides, was modified slightly for the methylation of OH-PCBs. The six congener mixture of OH-PCBs (8 nmol of each congener) was added to acetone (3 ml) followed by iodomethane (120 µl), and tetrabutylammonium hydroxide (60 µl). The samples were stoppered, vortexed and

placed in a 40°C water bath for 1.5 hours. The water level was kept above the level of solvent level in reaction vessels. Each sample was then transferred to a round bottom flask and reduced in volume (to approximately 1 ml), avoiding evaporation to dryness. Samples were brought up to 4 ml with hexane and extracted with sulfuric acid (2.0 ml, 0.5 M). The phases were allowed to separate and the organic layer was removed. The sulfuric acid phase was washed two more times with hexane (3 ml). Combined organic fractions were concentrated to approximately 1 ml and applied to a neutral alumina column (3.0 g, 0.5% deactivated, prewashed with 30 ml hexane). Analytes were eluted using 1:1 dichloromethane:hexane as the mobile phase. The first 30 ml of eluate were collected, concentrated to 550 µl and spiked with performance standard for analysis by GC-ECD. The performance standard for the derivatization reaction comparisons was 4-MeO-CB187.

2.5.2. Trimethylsilyldiazomethane

Using a similar procedure as above, the six congener mixture of OH-PCBs was added to a 1:9 methanol:acetonitrile mixture (3 ml). To this, trimethylsilyldiazomethane (5 µl, 2 M in hexanes) and N, N′-diisopropylethylamine (2.5 µl) were added with mixing. Reaction vessels were stoppered and the reaction was allowed to proceed for 2 hours. Water (2 ml) and concentrated sulfuric acid (1 ml) were added to each flask and the phases were allowed to separate before the organic layer was removed and purified on a neutral alumina column as described above.

2.5.3. Diazomethane

There are many methods used to generate diazomethane for methylation reactions. Production and storage of diazomethane in diethylether, which involves distillation/purification process, can lead to possible explosions. Long term storage of diazomethane in this manner may also lead to polymer formation through radical reactions that can result in additional unwanted peaks in a chromatogram. Simpler techniques have been developed such as diazomethane generation using an apparatus sold by Aldrich Chemicals (Milwaukee, WI, USA). This method proved to be unreliable since it did not generate enough diazomethane for the desired reactions. In addition, this method still involved handling of toxic nitroso precursors (e.g. Diazald®, *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemicals, Milwaukee, WI, USA), thus exposing the user to potentially harmful chemicals. A method of diazomethane preparation that allowed small quantities to be prepared, required no additional purification and resulted in few or no impurities that could interfere with the analysis was desirable. A method described by Blatt (221) filled all the desired prerequisites of diazomethane preparation. The method required synthesis of a non-commercially available precursor: nitrosomethylurea. Nitrosomethylurea is toxic but easy to handle. Since care must be taken when handling nitrosomethylurea to avoid skin contact or inhalation, its use is limited to a well-ventilated fume hood.

2.5.3.1. Preparation of Nitrosomethylurea

Methylamine (120 g, 40% aqueous solution, 1.55 moles) was weighed into a round bottom flask (1000 ml). Concentrated hydrochloric acid was added slowly to neutralize (~ 145 ml) the solution using pH paper as an indicator. Distilled water was added to bring the total weight to 500 grams. Urea (300 g, 3.41 moles) was added with boiling chips and the mixture gently refluxed for 2.75 hours and then vigorously for another 0.25 hours.

The reaction can be summarized as follows:

$$CH_3NH_3Cl + H_2NCONH_2 \rightarrow CH_3NHCONH_2 + NH_4Cl$$

The solution was allowed to cool overnight. The mixture was transferred to a separatory funnel through a funnel with glass wool to catch boiling chips. Sodium nitrite (110 g, 1.59 moles) was added to the separatory funnel and mixed until dissolved. This solution was then added dropwise (~ 1 hour) to a cooled and stirred mixture of ice (600 g) and concentrated sulfuric acid (100 g). The ice/acid mixture was in a beaker (3 L) with magnetic stirrer surrounded by an ice bath.

The reaction can be summarized as follows:

$$CH_3NHCONH_2 + HNO_2 \rightarrow CH_3N(NO)CONH_2 + H_2O$$

The foamy beige colored precipitate that formed was filtered off using a large Buchner funnel and filter paper. The precipitate was washed three times with ice cold water and dried under vacuum for 30 minutes. The precipitate was then transferred to a chemically cleaned glass jar, wrapped in aluminum foil, and placed in freezer for storage until needed. Total amount of solid recovered was 120 g resulting in a reaction yield of 75%.

2.5.3.2. Generation of Diazomethane

The solvent most often used for the collection and storage of diazomethane is diethyl ether. Using this solvent, a significant proportion of the less chlorinated OH-PCBs were ethylated. Therefore, hexane was chosen for the collection of diazomethane. Diazomethane was generated in a solution of hexane by adding nitrosomethylurea to a 1:1 bi-phasic mixture of sodium hydroxide aqueous solution (50% by weight) and hexanes in a Erlenmeyer flask (25 ml). The solid nitrosomethylurea sank to the bottom aqueous phase and reacted with the basic solution producing diazomethane, which dissolved into the hexane phase and saturated the organic phase, turning it the characteristic yellow color.

This technique is crude in that excess nitrosomethylurea is added until the user decides the color of the diazomethane/hexane solution is sufficient enough for the derivatization reaction. The top diazomethane/hexane phase is then isolated and used immediately for derivatization. Generally, 1-2 ml is used in the derivatization of samples and standards.

Diazomethane derivatization was then compared to the IPA method of methylation. The six congener mixture of OH-PCBs (8 nmol of each congener) was added to hexane (3 ml). Approximately 1 ml of the hexane:diazomethane solution was added to each . The tubes were then stoppered, vortexed and the reaction was allowed to proceed for 1.5 hours. The reaction mixture was then transferred to a 125 ml round bottom flask with hexane and evaporated to dryness to remove the excess diazomethane. The sample was immediately solvated with hexane and applied to a

neutral alumina column, following the steps as described for IPA. All samples were spiked with 4-MeO-CB187 prior to GC-ECD analysis.

2.6. Results and Discussion

The trimethylsilyldiazomethane procedure for methylation was unsuccessful, giving mostly trimethylsilylated derivatives instead of the desired methoxylated derivatives. This may have a purpose in the future by using trimethylsilylation as a means of separating closely eluting methoxylated PCBs in plasma.

IPA and diazomethane procedures both gave comparable degrees of derivatization. Five replicates of each method were compared. The results from the GC-ECD analysis are given in Table 2.3. Mean apparent relative response factors (ARRF) represent the ratio of the analyte area to that of 4-MeO-CB187. Increasing ARRFs indicate more methylation product formed during the methylation reaction.

Table 2.3 - Mean apparent relative response factors (ARRF) (± %CV, n=5) for each compound compared to 4-MeO-CB187 using GC-ECD mode of detection.

Compound	Retention Time (min)	IPA Mean ARRF	% CV	Diazomethane Mean ARRF	% CV
4'-OH-CB9	16.46	0.26	14	0.27	11
4-OH-CB9	16.72	0.11	11	0.071	27
3'-OH-CB61	22.66	0.33	8	0.23	27
2'-OH-CB106	22.83	0.77	14	0.31	33
4'-OH-CB61	23.44	0.12	12	0.089	25
4-OH-CB193	29.18	1.4	14	1.5	2

These results indicated that the IPA method of methylation gave as good or better yields of MeO-PCBs than diazomethane (203). The reproducibility of the IPA

method was also superior. These results were later confirmed by Rozemeijer *et al.* (215) for the methylation of metabolites from microsomal extracts. Although the IPA method may be superior for derivatization, more residual interferences were observed in the GC/ECD chromatogram of the final fraction relative to diazomethane. The use of diazomethane for derivatization resulted in very clean chromatograms using both ECD and GC-MS methods of detection. While IPA did produce a variety of extraneous peaks, interferences could likely be avoided by using a better clean up procedure or simply using MSD detection in the SIM mode. Under these conditions, IPA could replace diazomethane as a derivatization approach. The reagents for IPA are somewhat less toxic and easier to handle, but derivatization must still be completed in a well-ventilated fume hood. Another explanation for the higher %CV for diazomethane as compared to IPA could be the reaction time. Most literature descriptions of derivatization reactions using diazomethane allow one-hour reaction times, but no report for the derivatization of OH-PCBs has been described. Therefore, reaction time of the diazomethane methylation reaction was tested to ensure complete derivatization. It was found that over 3 hours of reaction time was necessary for most compounds while some lower chlorinated compounds required 24 hours of reaction time. This is likely the main reason for the variability in the ARRFs for the lower chlorinated compounds listed in Table 2.3.

Generation of the diazomethane via nitrosomethylurea was quick, simple and efficient derivatization for the higher chlorinated phenolic compounds commonly found in biota (5). Since the method allows control of the amount of diazomethane/hexane solution produced without the need for distillation or excessive

cleanup of the derivatized samples, it was chosen as the best method of derivatization for this application. All environmental samples were given 24 hours to react with diazomethane prior to further workup.

2.7. Analysis and quantitation of halogenated phenolic compounds

Before quantitative work is attempted, mass spectral properties of the compounds being analyzed need to be determined. When using selected ion monitoring (SIM), it is imperative to understand a compound's fragmentation patterns and responses to different mass spectral analysis conditions. Therefore, using some of the commercially available standards, ECNI mass spectral properties were investigated to establish the appropriate choice of ions for OH-PCB determination using their methyl ether derivatives.

2.7.1. Effects of Source Temperature on Response and Fragmentation

Selected compounds were analyzed by full scan ECNI (50-550 amu) mode to determine effect of source temperature on fragmentation patterns. Five source temperatures were tested. The compounds used included; 4′-MeO-CB107, 4-MeO-CB109, 4′-MeO-CB120, 4′-MeO-CB159, 4′-MeO-CB165, 4′-MeO-CB172, 3′-MeO-CB180, 4-MeO-CB187 and 4-MeO-CB193. Figures 2.1 to 2.4 show the source temperature effect on fragmentation of the penta, hexa and hepta chlorinated compounds. Each point is the summation of the abundances of the entire isotopic cluster and is expressed as a percentage of the total response of the compound at that

particular source temperature. Background spectra were subtracted prior to calculation of compound abundances.

As shown in Figures 2.1 to 2.4, higher source temperature in ECNI-MS resulted in increased abundance of fragment ions while lower source temperatures resulted in more molecular ion formation. This was previously shown for PCBs and other CHCs (222, 223). With increasing source temperature, the probability for dissociative electron capture increases and the probability of non-dissociative electron capture decreases (223, 224) resulting in increased fragmentation in the loss of $[M-CH_3]^-$ or $[M-HCl]^-$.

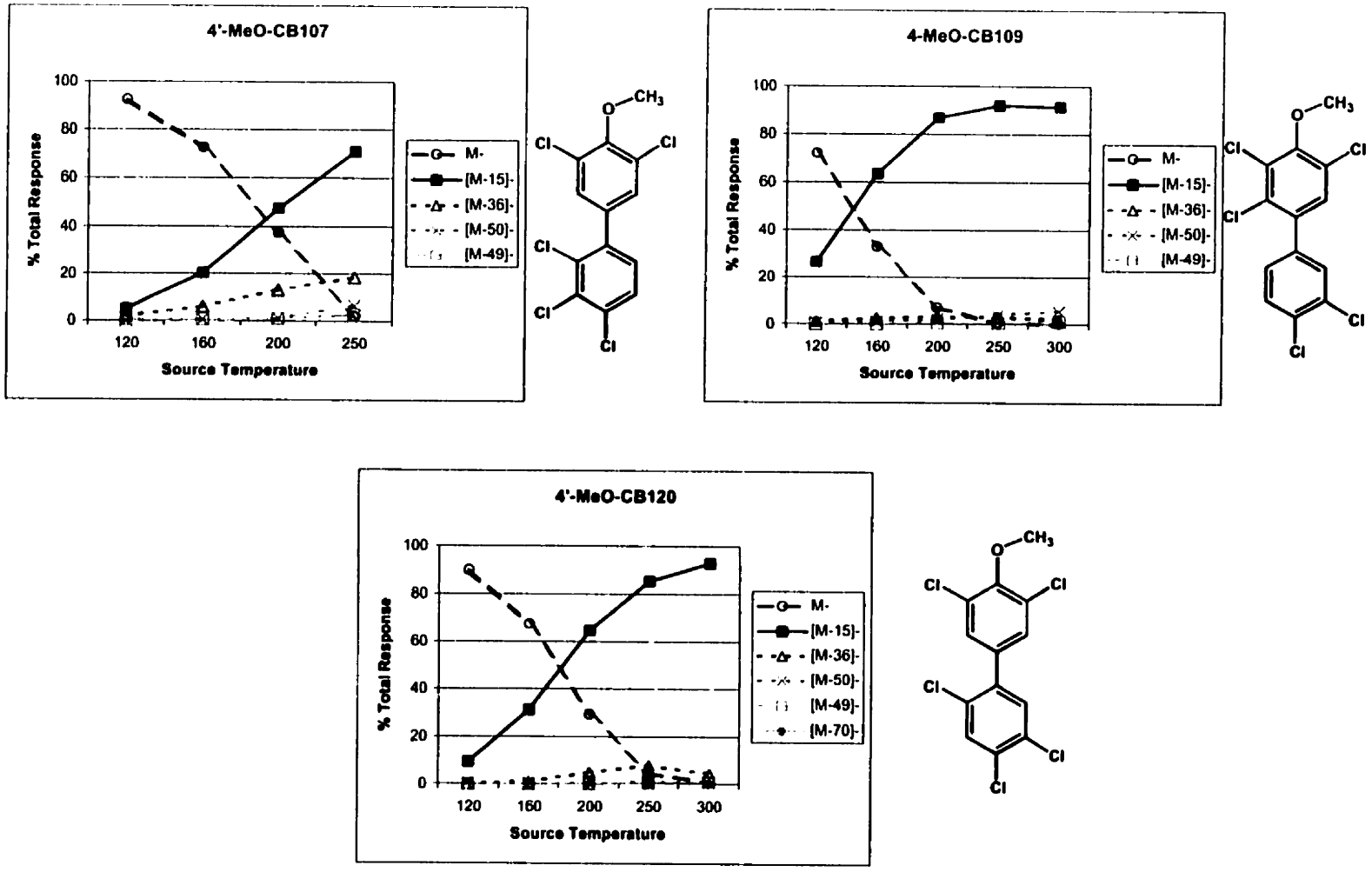
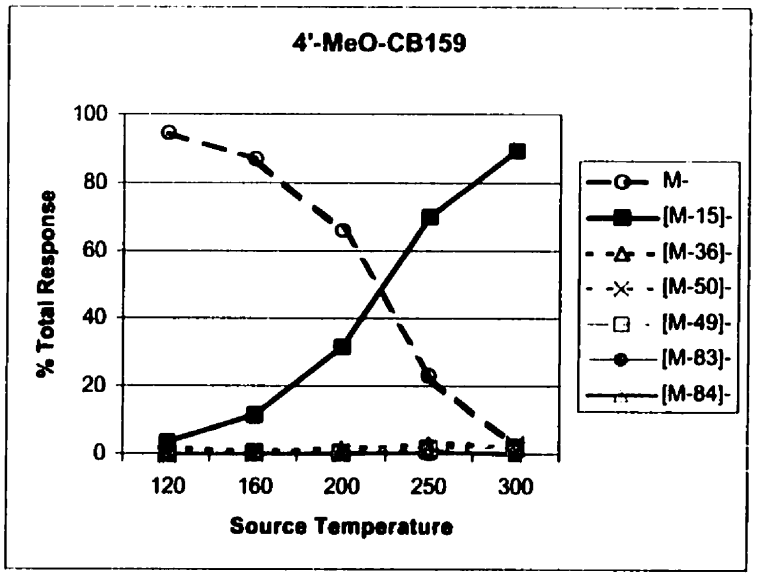
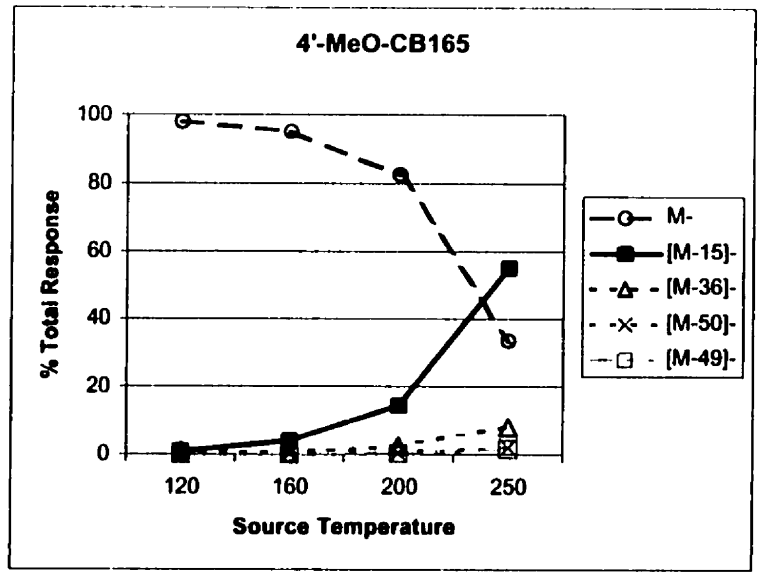
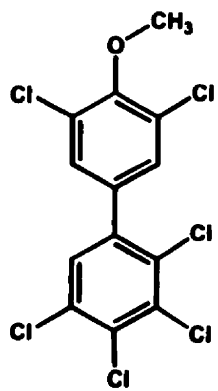


Figure 2.1 - Source temperature effects on the ECNI fragmentation of three selected pentachlorinated compounds. Each point represents the abundance of the entire isotopic cluster.







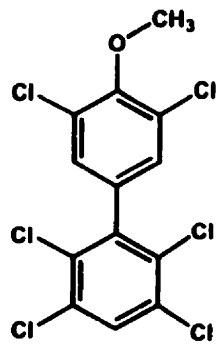
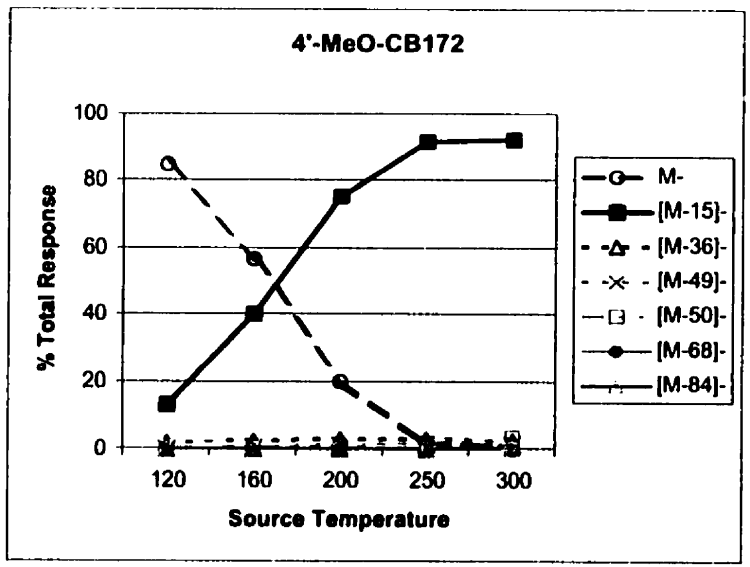
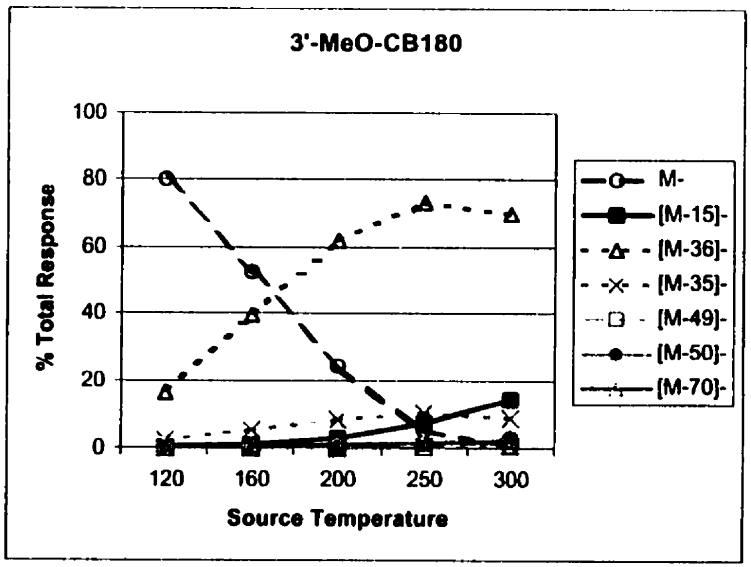
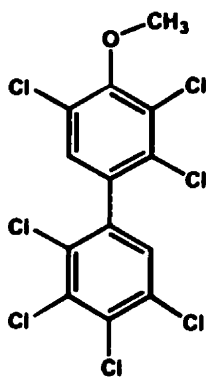


Figure 2.2 - Source temperature effects on the ECNI fragmentation of two selected hexachlorinated compounds. Each point represents the abundance of the entire isotopic cluster.







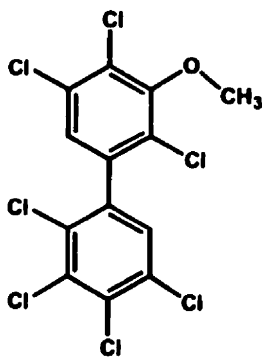
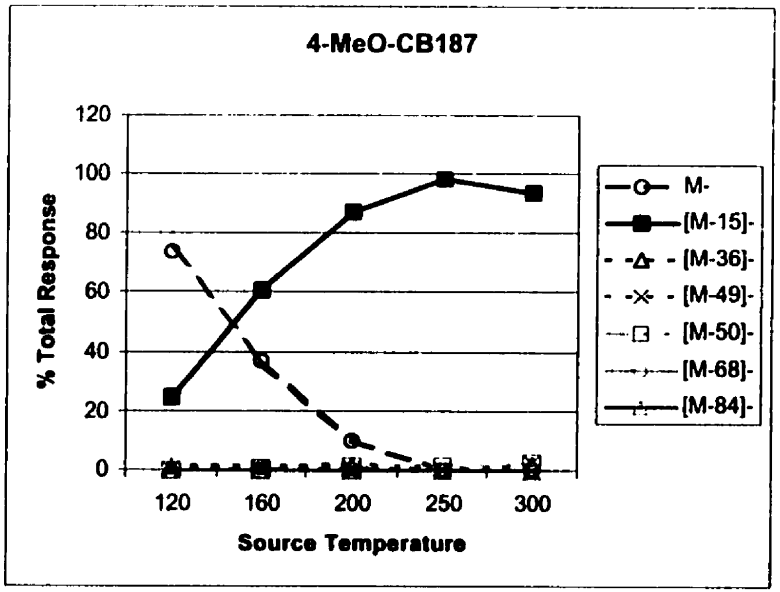
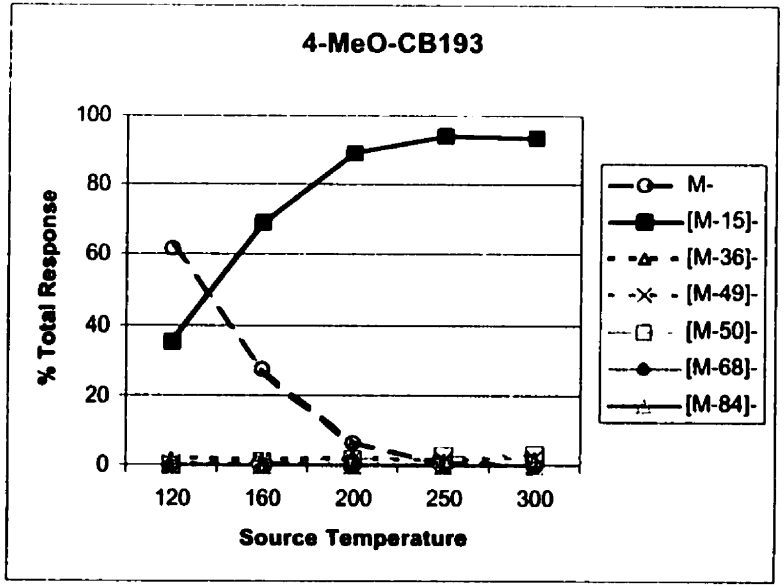
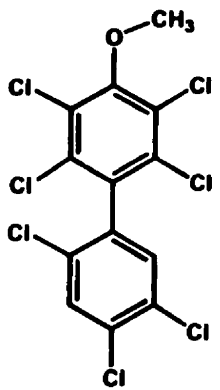


Figure 2.3 - Source temperature effects on the ECNI fragmentation of two selected heptachlorinated compounds. Each point represents the abundance of the entire isotopic cluster.







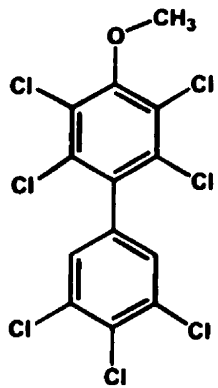


Figure 2.4 - Source temperature effects on the ECNI fragmentation of two selected heptachlorinated compounds. Each point represents the abundance of the entire isotopic cluster.

The general trend for all OH-PCBs tested is a rapid decrease in the molecular ion cluster with a concomitant increase in $[M-CH_3]^-$ cluster with increasing source temperature. The $[M-CH_3]^-$ fragment was also the main fragment seen for most para substituted MeO-PCBs in a previous study where the source temperature was set at 150°C (211). The only exception to the increase in the $[M-CH_3]^-$ cluster involved the fragmentation of the only meta substituted compound tested, 3'-OH-CB180. This compound showed an increase in the loss of HCl^- rather than $CH_3^{\cdot}$. This type of fragmentation commonly occurs in chlorinated aromatic compounds (222, 224-227) and was previously observed for MeO-PCBs (211). Compounds with para substituted MeO groups and meta chlorines (3,5 position) seem to have a more stable molecular ion since it takes higher source temperatures (> than 180°C) to cause loss of $CH_3^{\cdot}$. This is in contrast to para substituted MeO groups with meta chlorines (3,5 position) and at least 1 ortho chlorine where the $[M-CH_3]^-$ fragment becomes dominant at lower source temperatures (< than 160°C). These trends in fragmentation need to be verified for more congeners in a more comprehensive study to confirm this structure specific fragmentation.

All fragment identities are listed in Table 2.4. Most of these ions were previously noted by Bergman *et al.* (211). The only ions in need of interpretation are those that involve addition of hydrogen before fragmentation. These types of ions are formed via a secondary process where an electron is captured by a species, which was generated by a heterogeneously catalyzed hydrogenation of the analyte on surfaces of the ion source (228). This type of process has been documented previously for the

mass spectra of methylsulfone metabolites of PCBs (227) and hexachlorocyclopentadiene derivatives (224). This process has also been shown be quite erratic as it varies with source temperature, pressure and surface condition and therefore does not produce stable monitoring ions.

Table 2.4 - Fragment ions and their presumed identities for the ECNI-MS of MeO-PCBs.

Ion Cluster	Fragment Loss
M-35	M-Cl
M-36	M-HCl
M-49	$M+H-CH_3Cl$
M-50	$M- CH_3Cl$
M-68	[M+H-Cl] x 2
M-70	[M-Cl] x 2
M-84	$[M+H-CH_3Cl_2]$

As shown above, source temperature is particularly important in the selection of monitoring ions in SIM mode. This was also shown to be true for other CHCs by Stemmler and Hites (225).

Source pressure was also tested for effects on fragmentation. Pressure, as adjusted by reagent gas, was shown to have little effect on fragmentation patterns of MeO-PCBs but did affect overall response. This was also shown to be the case for hexachlorocyclopentadiene derivatives by Stemmler and Hites (224). Reagent gas pressure is generally set to maximize the presence of the reactive $C_2H_5^+$ ion prior to tuning the mass spectrometer, therefore data for source pressure effects on fragmentation on OH-PCBs is not shown. The reagent gas pressure was generally set at 2.5×10^{-4} torr as measured at the source inlet.

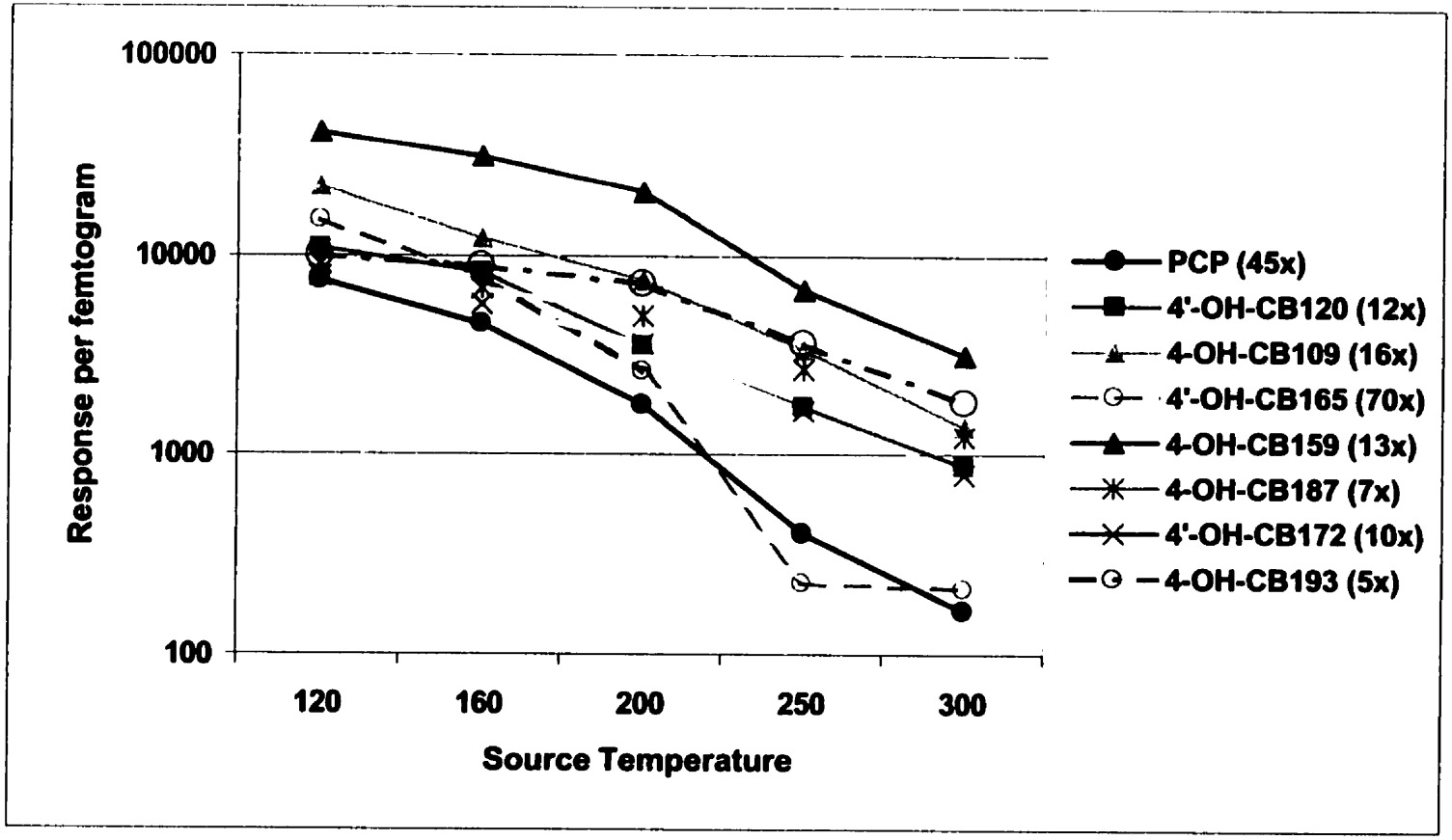


Figure 2.5 - Effect of source temperature on the total ion response of selected phenolic compounds. Decrease in total response from 120°C to 300°C shown in parentheses.

Source temperature effects on total ion chromatograms (TIC) of selected halogenated phenolic compounds were tested at different source temperatures ranging from 120°C to 300°C. Background spectra were subtracted from each analyte's spectra prior to TIC calculations. The effects of source temperature can be seen in Figure 2.5. All compounds decreased in response ranging from 5 to 70 fold. Similar to the findings described by Stemmler and Hites (224), the source temperature giving the highest response was 100°C (not shown). The instrument heater assembly in the instrument was unable to maintain a constant source temperature of 100°C and run times increased dramatically while waiting for temperature stabilization. Therefore, the chosen source temperature was 140°C. The increased sensitivity of a lower source temperature comes at a cost to the operator, as frequent cleaning of the source assembly is required. Increased contamination at lower source temperatures was also noted by Oehme *et al.* (229) in the analysis of other common CHCs.

2.8. Characterization of Unknown Phenolic Compounds -Application to Polar Bear Plasma

Many compounds in the phenolic compound fraction remain unidentified (201) and new standards are being synthesized regularly. Therefore, a large pool of polar bear plasma was extracted and characterized by full scan ECNI mass spectrometry. Comparison of retention times on GC columns with different polarities confirmed identities of compounds where standards were available. Unknown compounds were characterized by full scan ECNI and ions were chosen so that relative concentrations could be monitored until standards became available.

Pooled polar bear plasma (33 g) was extracted as described by Bergman *et al.* (5) and analyzed for the presence of halogenated phenolic compounds using the optimized mass spectral conditions described above. The polar bear phenolic compound extract was injected on four different GC columns to confirm the identity of compounds against authentic standards. Main congeners that remained unidentified were characterized by their full scan mass spectra.

Four mixtures of MeO-PCBs (Table 2.2) were injected onto four different GC columns along with the extracted phenolic compound fraction from the pooled polar bear plasma. The pooled polar bear sample was spiked with performance standard, 4′-Me-4-MeO-CB112, prior to injection. With each column, multiple injections were made to optimize the GC temperature program for maximum resolution of standards prior to the analysis of all the standard mixtures and the sample.

In all previous plasma samples analyzed, the phenolic fraction consistently contained PCP and the dominant OH-PCB congener, 4-OH-CB187. Both compounds were available as ^{13}C labeled standards. Since these two compounds have very different retention times and are found on opposite ends of the chromatogram, a retention time index (RI) was developed so that an unknown compound can be identified and characterized between laboratories even if authentic standards are not available. The retention time index was calculated as follows (T = retention time):

$$RI = \frac{(T_x - T_{PCP})}{(T_{4\text{-OH-CB187}} - T_{PCP})}$$

Table 2.5 - Retention times and retention time indices for OH-PCBs on four different GC columns. Highlighted congeners were positively identified in polar bear plasma (PS=4′-Me-4-MeO-CB112).

DB-1						DB-5					
Compound	R_t	RI	Compound	R_t	RI	Compound	R_t	RI	Compound	R_t	RI
PCP	14.184	0.000	4-OH-CB146	22.339	0.831	[redacted]	14.387	0.000	[redacted]	22.818	0.825
4'-OH-CB61	18.984	0.490	3'-OH-CB138	23.126	0.910	4'-OH-CB61	19.417	0.493		23.806	0.922
3-OH-CB85	20.819	0.675	4'-OH-CB130	23.198	0.918	3-OH-CB85	21.295	0.676		23.894	0.931
4'-OH-CB120	21.111	0.706	3'-OH-CB187	23.653	0.964	[redacted]	21.459	0.693		24.227	0.963
4'-OH-CB104	21.373	0.733	4'-OH-CB175	23.873	0.986	4'-OH-CB104	22.052	0.751	4'-OH-CB175	24.484	0.988
3-OH-CB118	21.746	0.770	4-OH-CB187	23.996	1.000	[redacted]	22.244	0.769		24.604	1.000
4'-OH-CB107	21.777	0.774	4'-OH-CB159	24.251	1.026	[redacted]	22.310	0.775		24.886	1.028
4-OH-CB109	21.790	0.774	3'-OH-CB180	25.701	1.173	4'-OH-CB107	22.320	0.777		26.375	1.174
PS	22.053	0.802	4'-OH-CB172	25.770	1.181	4-OH-CB188	22.466	0.791		26.468	1.182
4-OH-CB188	22.056	0.801	4-OH-CB193	26.194	1.223	PS	22.532	0.799		26.870	1.222
3-OH-CB153	22.297	0.826				[redacted]	22.752	0.819			

DB-1701						DB-210					
Compound	R_t	RI	Compound	R_t	RI	Compound	R_t	RI	Compound	R_t	RI
PCP	15.818	0.000	4'-OH-CB104	32.866	0.957	PCP	16.533	0.000	4-OH-CB146	31.715	0.893
4'-OH-CB61	30.031	0.798	3'-OH-CB138	33.173	0.974	4'-OH-CB61	25.690	0.539	3'-OH-CB187	32.864	0.961
3-OH-CB85	30.119	0.803	3'-OH-CB187	33.195	0.975	3-OH-CB85	29.429	0.759	4'-OH-CB175	33.215	0.982
4'-OH-CB120	30.162	0.805	4'-OH-CB130	33.262	0.979	4-OH-CB188	29.702	0.775	3'-OH-CB138	33.237	0.983
4-OH-CB188	30.920	0.848	4'-OH-CB175	33.415	0.988	4'-OH-CB120	29.806	0.781	4'-OH-CB130	33.392	0.992
PS	31.503	0.882	4-OH-CB187	33.636	1.000	PS	30.339	0.813	4-OH-CB187	33.522	1.000
4'-OH-CB107	31.525	0.882	4'-OH-CB159	34.129	1.028	4'-OH-CB104	30.574	0.827	4'-OH-CB159	34.664	1.068
3-OH-CB118	31.526	0.882	3'-OH-CB180	35.505	1.105	4'-OH-CB107	31.371	0.874	3'-OH-CB180	36.263	1.161
4-OH-CB109	31.570	0.884	4'-OH-CB172	35.591	1.110	3-OH-CB118	31.458	0.879	4'-OH-CB172	36.420	1.171
3-OH-CB153	31.811	0.898	4-OH-CB193	36.093	1.138	4-OH-CB109	31.524	0.882	4-OH-CB193	37.052	1.208
4-OH-CB146	31.899	0.903				3-OH-CB153	31.590	0.886			

Results of the multiple column experiment are given in Table 2.5. The best resolution was achieved using the DB-5 column. The DB-5 column also allows quicker analysis time due to higher temperature resistance as compared to higher polarity columns. Since the DB-5 column is used for PCB and CHC analyses, this is the column of choice for the phenolic compound analyses.

Fourteen compounds (including PCP) were identified by comparison of retention indices (RI) as well as full scan spectra with authentic standards. Positive identification criteria were an RI difference of less than 0.001 on all four columns. The identified compounds have been highlighted in Table 2.5. It is interesting to note that only 4-OH-CB109, and not the co-eluting 4′-OH-CB107 (on DB-5), was found in polar bear plasma. Both of these peaks have previously been identified in human and Baltic seal plasma (5). In that same study, Aroclor dosed rats were similar to polar bear as they had only the single congener, 4-OH-CB109, in their plasma (5).

All compounds identified in polar bear plasma have previously been identified in human plasma or Baltic seal (5) except 4-OH-CB193. This newly identified compound was actually used as an internal recovery standard in the above study. This compound may be unique to the polar bear due to its uniquely high metabolic capability (230). It is also possible that it remained below detection in the previous study (5). Both CB191 and CB193 can form 4-OH-CB193 through NIH shift or direct insertion mechanisms of hydroxylation. These precursor PCBs constitute very small proportions of the Aroclor 1260 technical mixture (0.17 and 0.53%, respectively) (9), so it is likely that the amount of 4-OH-CB193 is very small.

An unknown compound was observed at an earlier retention time prior to any OH-PCB elution. It was frequently the major peak observed in other polar bear plasma samples. The inclusion of two major ions (324, 326) from its full scan spectra in the SIM method allowed the compound to be monitored. Its mass spectra showed a hexachlorinated isotope cluster, therefore initial concentrations were calculated based on the response factor from the hexachlorinated OH-PCBs. Chapter 4 describes the elucidation of the unknown compound's identity and some of its toxicological properties in polar bear plasma.

Table 2.6 - List of the twelve most abundant unidentified OH-PCBs. Chlorine substitution, MeO positions and RIs are shown along with hypothesized identities for three compounds.

Unknown Compounds				
	Compound	OH-substitution	R_t	DB-5 RI
1	Cl_5	meta	20.528	0.601
2	Cl_6	para	22.594	0.803
3	Cl_6	meta	23.785	0.920
4	Cl_6	para	24.114	0.952
5	Cl_6	para	24.902	1.029
6	Cl_6	para	25.087	1.047
7	Cl_8	para	25.699	1.107
8	diOH-Cl_7	para	27.559	1.289
9	Cl_8	para	27.814	1.314
10	Cl_8	para	27.999	1.332
11	4,4'-diOH-CB202	para	28.854	1.416
12	4'-OH-CB208	para	29.131	1.443

There remained many unidentified compounds in the polar bear plasma chromatogram. The main 12 compounds were characterized by full scan spectra. Figure 2.6 shows the entire OH-PCB chromatogram with all identified and

characterized unidentified compounds (numbered). Table 2.6 shows the unknown OH-PCB's RIs and the likely number of chlorines on these compounds as determined by their assumed molecular ion isotopic cluster.

From results described above and those described by Bergman *et al.* (211), para and meta MeO substitutions can be deduced from ECNI-MS fragmentation patterns of the MeO-PCBs. Compounds that have large M-CH_3 clusters in their mass spectra are generally para substituted while those with low or absent M-CH_3 are usually meta substituted. Meta substituted compounds generally have low molecular ion abundances as well (211). No ortho substituted compounds have ever been found in plasma (5, 151, 201). Figures 2.7 – 2.12 show the ECNI mass spectra for the unknown OH-PCBs found in polar bear plasma.

The first unknown compound has five chlorines, the correct M^- for a MeO-PCB from its molecular ion isotopic cluster, and gives an abundant [M-HCl] without any M-CH_3 fragment. This is indicative of a meta substituted methoxy group (211) as described above for the fragmentation of 3'-OH-CB180. The same observations hold for the third unknown compound, which is likely derived from a meta substituted hexachlorinated methylated biphenylol.

Unknown 8 is one of the largest methylated biphenyldiols found in polar bear plasma. The mass spectrum shows a molecular weight similar to a methylated octachlorobiphenylol (beginning at 4 amus less) but has a heptachloro isotope cluster. The fragmentation is similar to other methylated chlorobiphenylols having abundant [M-CH_3], [M-Cl] and [M-CH_3Cl] but also has abundant loss of hydrogen products as displayed by [M-HCl] fragment and the hydrogenation product [M-49].

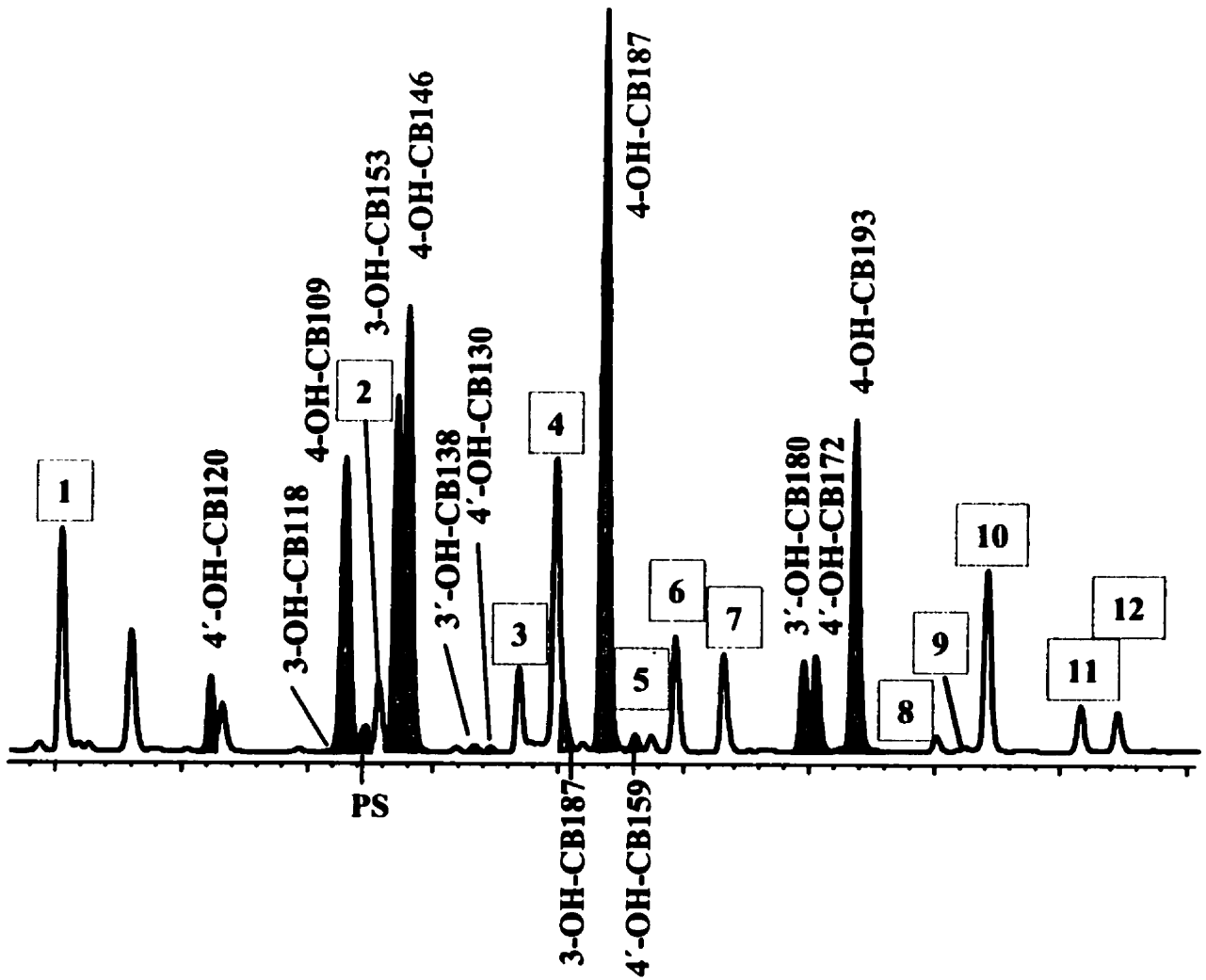


Figure 2.6 - Fullscan ECNI chromatogram of the MeO-PCB fraction from a pooled polar bear sample. All confirmed structures are labeled and shown in gray. Numbers indicate unknown compounds that were characterized by a fullscan spectra (P.S. = performance standard).

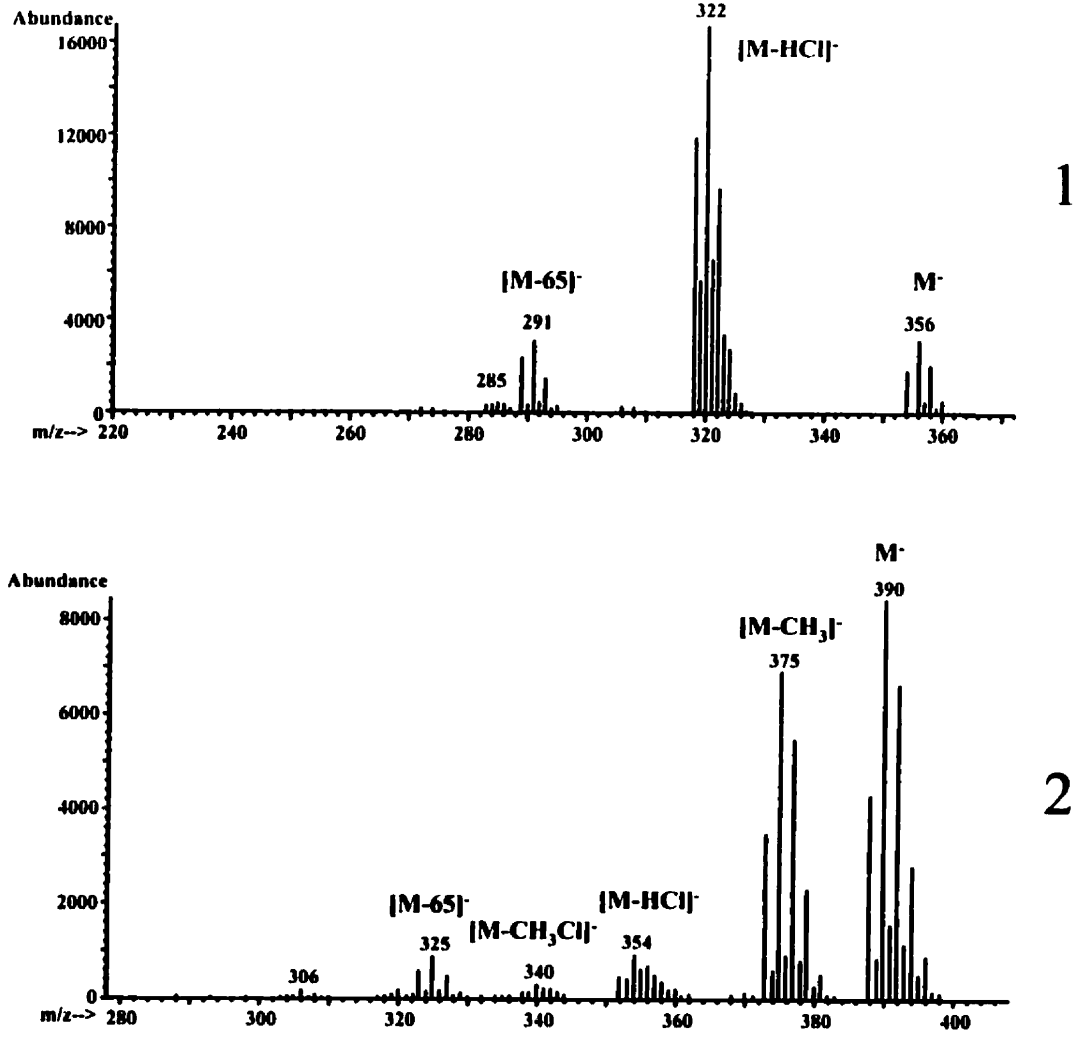


Figure 2.7 - ECNI mass spectra for unknown OH-PCBs 1 and 2.

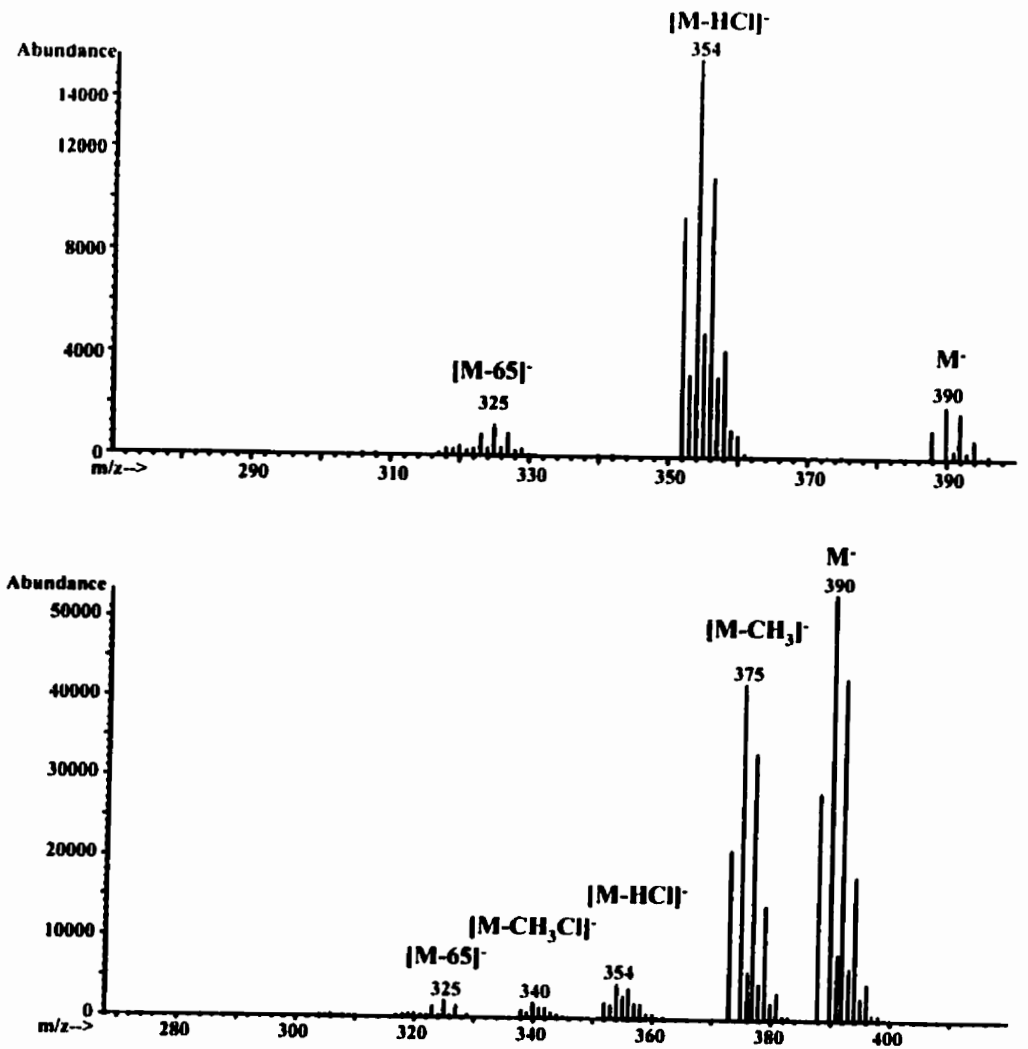


Figure 2.8 - ECNI mass spectra for unknown OH-PCBs 3 and 4.

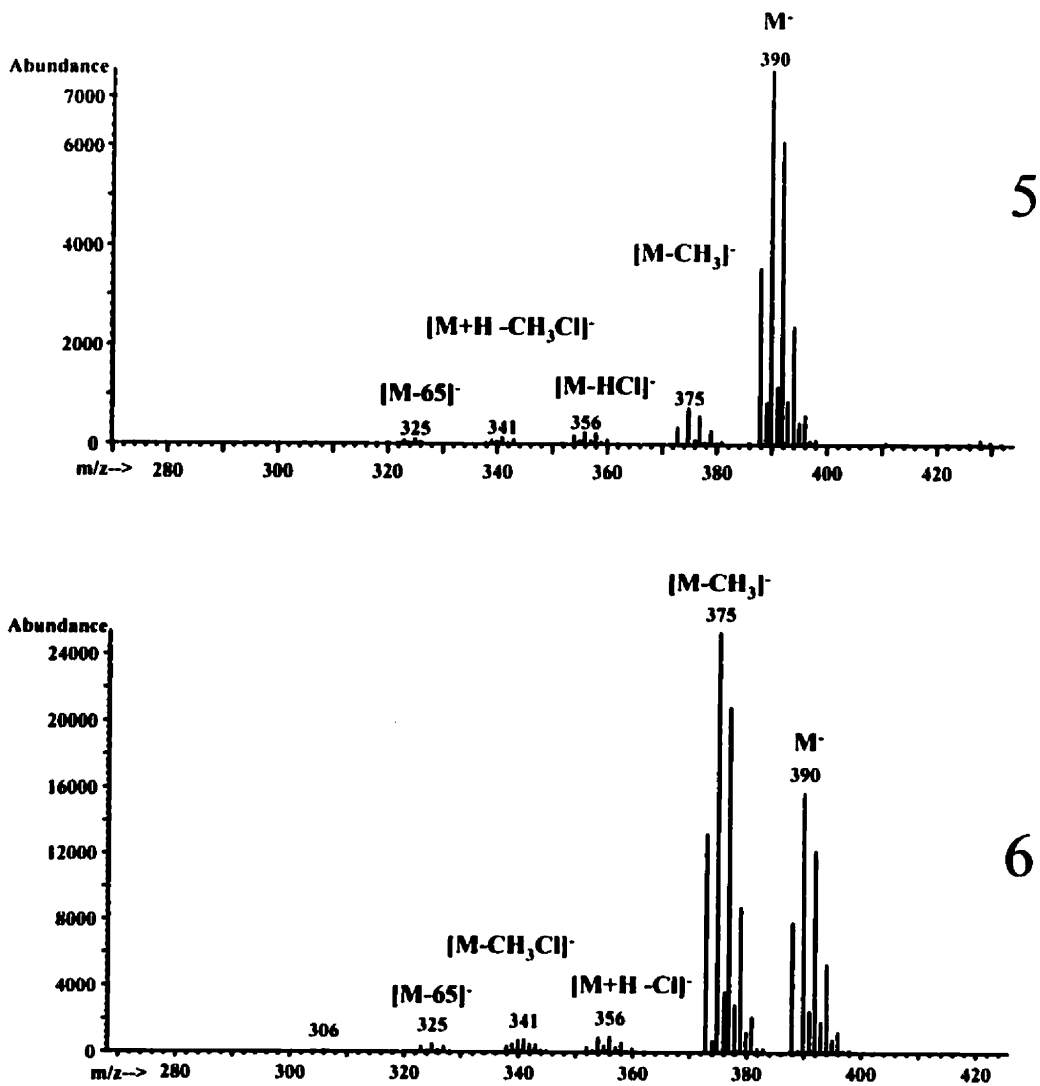


Figure 2.9 - ECNI mass spectra for unknown OH-PCBs 5 and 6.

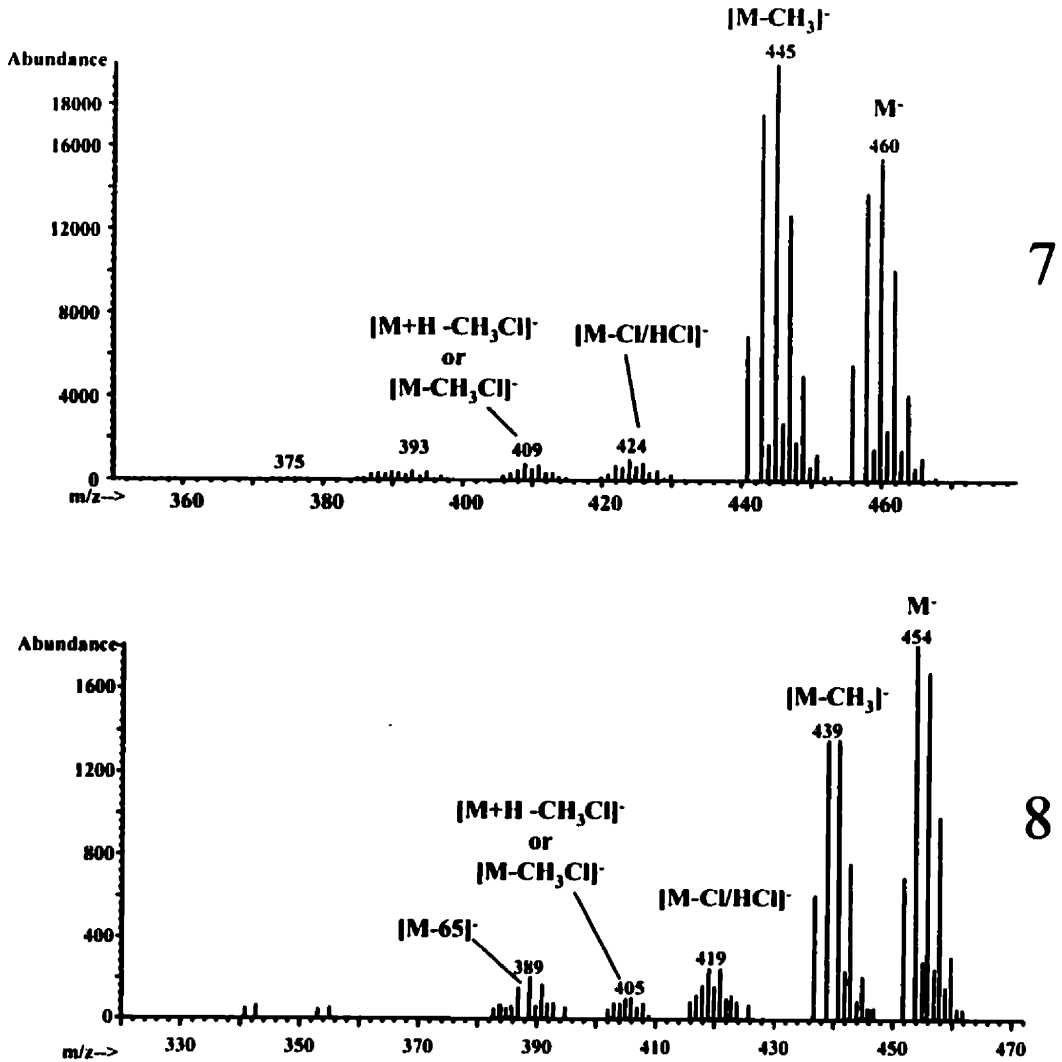


Figure 2.10 - ECNI mass spectra for unknown OH-PCBs 7 and 8.

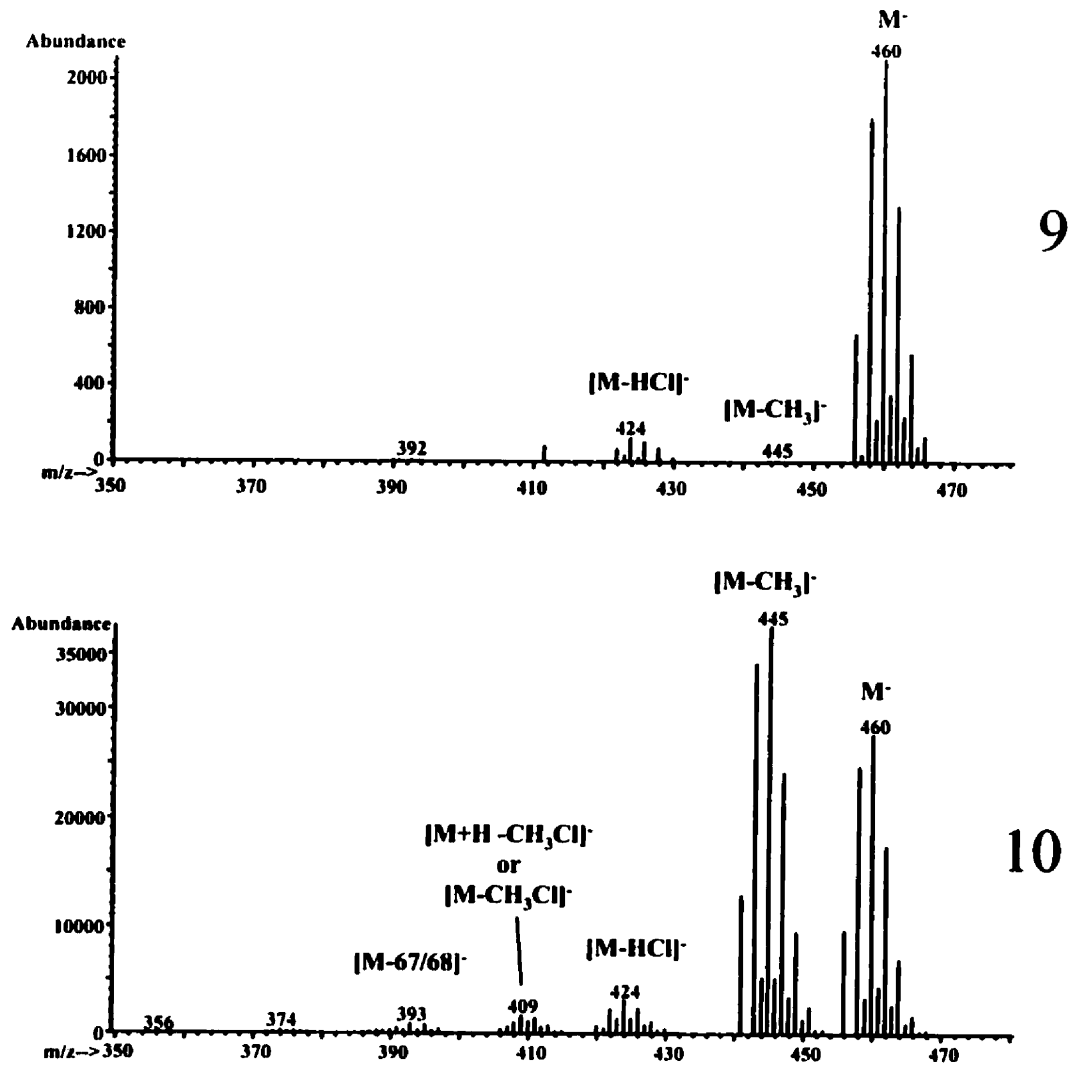


Figure 2.11 - ECNI mass spectra for unknown OH-PCBs 9 and 10.

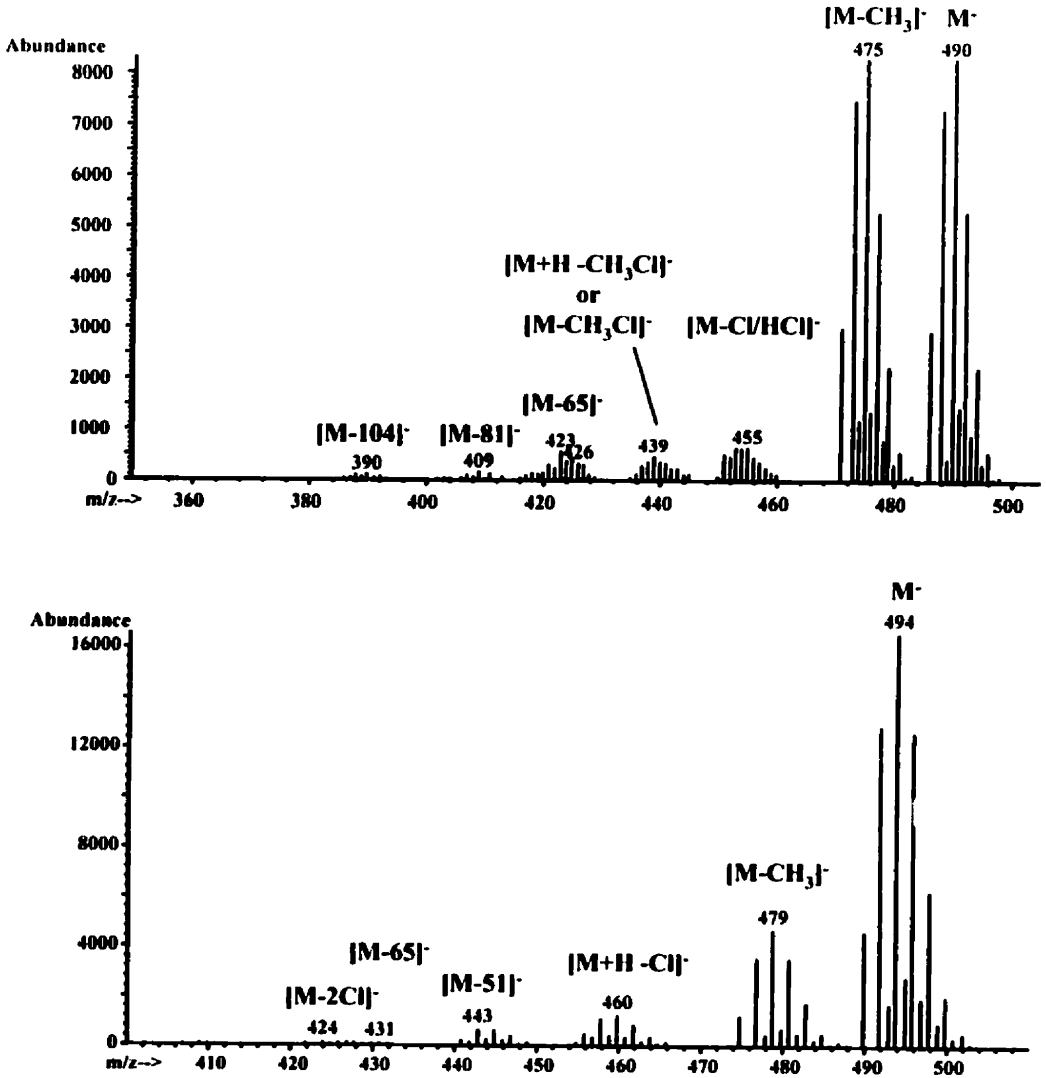


Figure 2.12 - ECNI mass spectra for unknown OH-PCBs 11 and 12.

Table 2.7 - Selected ion monitoring program designed for the detection of OH-PCBs in polar bear plasma using GC program for DB5 column in Table 2.1.

Retention Time Range (minutes)	Compound	Target/ Qualifier	Retention Time Range (minutes)	Compound	Target/ Qualifier
10.0 - 17. 0	PCP	280, 282	22.4 - 24.20	meta-OH-Cl_5CB	320, 322
	^{13}C - PCP	286, 288		OH-Cl_5CB	356, 358
	TeCl-phenol	246, 244		diOH-Cl_5CB	386, 388
	2,4,6-tribromophenol	79, 81		4'-Me-4-MeO-CB112	368, 370
				OH-Cl_6CB	390, 392
17.0 - 20.5	4-OH-HpCS	324, 326		diOH-Cl_6CB	420, 422
	OH-Cl_4CB	320, 322		OH-Cl_7CB	424, 426
	diOH-Cl_4CB	350, 352		diOH-Cl_7CB	454, 456
	OH-Cl_5CB	356, 358		Bromine Trace	79, 81
	diOH-Cl_5CB	386, 388			
	OH-Cl_6CB	390, 392	24.2 - 26.8	meta-OH-Cl_5CB	320, 322
	diOH-Cl_6CB	420, 422		OH-Cl_5CB	356
	OH-Cl_7CB	424, 426		OH-Cl_6CB	390, 392
	Bromine Trace	79, 81		diOH-Cl_6CB	420, 422
				meta-OH-Cl_7CB	396
20.5 - 22.4	OH-Cl_4CB	320, 322		OH-Cl_7CB	424, 426
	diOH-Cl_4CB	350, 352		diOH-Cl_7CB	454, 456
	OH-Cl_5CB	356, 358		OH-Cl_8CB	460, 458
	diOH-Cl_5CB	386, 388		^{13}C - 4-OH-CB187	436, 438
	OH-Cl_6CB	390, 392		^{13}C - 4'-OH-CB172	436, 438
	diOH-Cl_6CB	420, 422		^{13}C - 4-OH-CB159	402, 404
	OH-Cl_7CB	424, 426		Bromine Trace	79, 81
	diOH-Cl_7CB	454, 456			
	^{13}C - 4-OH-CB120	368, 370	26.8 - 30	OH-Cl_6CB	390, 392
	Bromine Trace	79, 81		diOH-Cl_6CB	420, 422
				OH-Cl_7CB	424, 426
22.4 - 24.20	meta-OH-Cl_5CB	320, 322		diOH-Cl_7CB	454, 456
	OH-Cl_5CB	356, 358		OH-Cl_8CB	460, 458
	diOH-Cl_5CB	386, 388		diOH-Cl_8CB	490, 488
	4'-Me-4-MeO-CB112	368, 370		OH-Cl_9CB	494, 492
	OH-Cl_6CB	390, 392		Bromine Trace	79, 81
	diOH-Cl_6CB	420, 422			
	OH-Cl_7CB	424, 426			
	diOH-Cl_7CB	454, 456			
	Bromine Trace	79, 81			

The fragmentation observed for unknown 8 is also seen for unknown 11. Unknown 11 is likely a dimethoxylated octachlorobiphenyl as determined by its fragmentation and isotopic cluster. Unknown 11 has been tentatively identified as 4,4′-diOH-CB202 as described by Letcher *et al.* (231).

The last eluting compound, unknown 12. is the only methoxylated nonachlorobiphenyl in polar bear plasma. Its identity is likely 4′-OH-CB208 as described by Letcher *et al.* (231). The para substitution of the methoxy group is deduced from the abundant M-CH_3 fragment and the number of chlorines from the nonachloro isotope cluster.

The other major compounds are listed in Table 2.6 and their fragmentation described is shown in Figures 2.7 to 2.12. As seen from Table 2.6, the compounds are predominantly hexachlorinated with a para substituted methoxy group.

Many compounds are present in the polar bear plasma samples that have not been included, as they were not found at high enough concentrations to acquire full scan spectra. There were penta and multiple hexa and heptachlorinated methoxy-biphenyls, therefore additional ions were monitored in the final methodology so that these compounds could be monitored and semi-quantitated.

An SIM method for monitoring OH-PCBs and other halogenated phenolic compounds was created using the optimized mass spectrometry conditions, choosing the main ions found in the available commercial standards and unknown chlorinated compounds found in polar bear plasma. Table 2.7 lists the ions and the retention time ranges that were monitored. Br^-, which is a major ion in the ECNI spectra of brominated hydrocarbons, was monitored throughout the chromatogram to determine

the presence of brominated compounds in polar bear plasma. Recently, hydroxylated metabolites of polybrominated diphenyl ethers were found in fish samples (207), making it important to monitor their possible presence in polar bear. However, no standards are currently available for the hydroxylated brominated diphenyl ethers. Semi-quantitation of MeO-PCBs can be done using average homologue response factors of authentic standards in that particular homologue group. However, ECNI fragmentation, and therefore response factors, of aromatic chlorine positional isomers varies much more than in EI, and this approach is crude at best. Tetrachlorinated MeO-PCBs were included in the mass spectral program but due to standard availability, none were detected or quantitated any samples.

2.9. Detection Limits

Using the SIM method described above detection limits for the main commercially available standards were determined. A serial dilution (1000, 500, 200, 100, 50, 5, 1, 0.25 and 0.1 pg/μl) of standards were injected on the mass spectrometer and selected ions monitored using optimal mass spectral conditions (source temperature of 140°C, methane pressure of 2.5 x 10^{-4} torr) and the SIM program in Table 2.7 for selected compounds. Noise was determined from the baseline deviations for the ion monitored for a given compound and instrument detection limits (IDLs) were set at three times the S/N ratio. Table 2.8 lists the compounds and their IDLs and linear range. The lowest concentration standard for each standard was 0.1 pg/μl, thus some linear ranges extended past the lowest level tested. The upper linear range was

established by removing the highest concentration standard until the regression coefficient (r^2) was greater than 0.97.

As demonstrated in Table 2.8, IDLs were quite variable among compounds, ranging from 21 ppt to 3 ppb. Response in electron capture negative ionization is higher for the higher chlorinated compounds because electronegativity increases with number of chlorines. As seen in Table 2.8, detection limits of many of the pentachloro compounds were much higher than hexa and heptachloro compounds. The response of tetrachloro compounds was also very poor using GC-ECNI-MS detection and thus ions were monitored but not quantitated in samples. The MeO substitution also affects the response. This is seen by comparing the ortho substituted 6-OH-CB86 and 6′-OH-CB93 to the para substituted counterparts, 4′-OH-CB86 and 4′-OH-CB93. The ortho substituted congeners had IDLs 21 and 2 times lower than the para substituted congeners. This is due to the para compounds undergoing fragmentation more readily, thus decreasing the response of the molecular ion monitoring ions (356, 358). Although there is variation for response among compounds with different number of chlorines, instrument detection limits of sub pg/μl levels are likely adequate for the detection of OH-PCBs in biota. Letcher *et al.* (231) has noted levels of sum OH-PCBs in plasma to be between 5-50% those of sum PCBs which are usually well above those levels in non-exposed humans.

Table 2.8 – Instrument detection limits (IDL) and linear range of selected compounds using the SIM program described in Table 2.7.

#Cl	Compound	fg/ul IDL	pg/ul Linear Range	r^2
5	PCP	21	0.1-100	0.999
5	3'-OH-CB85	462	0.5-200+	0.993
5	6-OH-CB86	143	0.1-200+	0.991
5	4'-OH-CB86	3000	5-200+	0.999
5	6'-OH-CB93	750	1-200+	0.996
5	4'-OH-CB93	1304	1-200+	0.992
5	3-OH-CB118	138	0.1-100	0.996
5	4'-OH-CB107	225	0.1-100	0.987
5	4-OH-CB109	90	0.1-100	0.969
5	4'-OH-CB120	133	0.1-500+	0.999
5	4'-OH-CB121	2500	1-200+	0.983
5	4'-OH-CB130	196	0.1-100	0.999
6	4'-OH-CB159	57	0.1-250+	0.975
6	4'-OH-CB165	66	0.1-100	0.996
7	4'-OH-CB172	79	0.1-200+	0.998
7	3'-OH-CB180	78	0.1-100	0.999
7	4-OH-CB187	70	0.1-250+	0.987
7	4-OH-CB193	155	0.1-100	0.974

In summary, a SIM method using ECNI mass spectrometry was developed for the quantitation of halogenated phenolic compounds. Three derivatization procedures were tested, and methylation by diazomethane was chosen and optimized. Retention indices of known and unknown halogenated phenolic compounds were determined on four GC columns of different polarity to aid in identification. ECNI mass spectrometry conditions were optimized for maximum response and the IDLs established for application to authentic samples. Unknown compounds (lacking authentic standards) in polar bear plasma were characterized by full scan MS analysis and their ions monitored so that they can be identified and quantitated. The ECNI detection limits for the main phenolic compounds were as low or lower than ECD detection limits (not

shown) allowing better quantitation capability versus ECD methods used in OH-PCB determination in other studies (5). ECNI mass spectrometry also allowed better peak characterization and identification of co-eluting peaks by selection of the appropriate ions. Since many compounds could not be positively identified and the remaining phenolic compound fraction is not completely characterized, many co-eluting peaks may still exist. ECNI-MS peak characterization and identification also provided a means of semi-quantitation of the identified compounds.

Chapter 3. Extraction, Lipid Weight Determination and Quantification of Halogenated Phenolic Compounds in Polar Bear Plasma

3.1. Background

Extraction of the phenolic fraction from environmentally exposed animals is not new (200). Jansson *et al.* were the first to extract PCB metabolites from the excreta of Baltic Guillemot and seals (200). PCB metabolites have also been analyzed in the excreta of Aroclor dosed animals (232-234). Recently, it was determined that phenolic metabolites can also be extracted from blood (5). The methodology used for the extraction of phenolic metabolites in blood was very similar to the methodology described for extraction of excreta.

The original methods involved an initial extraction step involving hexanes followed by lipid removal using gel permeation chromatography. The extract was then partitioned using alkaline methanol to ionize the phenolic compounds and separate them from the neutral compounds. The alkaline phase was then acidified, back extracted and derivatized prior to further purification steps. The main difference for the extraction of phenolic compounds from blood as compared to excreta is the deproteination step required to decrease the amount of protein binding in the protein rich blood tissue (5).

Therefore, from these methods, a revised method for the analysis of halogenated phenolic compounds in plasma was developed (5, 200, 208). The clean up method

maximized extraction of both neutral (PCBs and other CHCs) and acidic components and then separated the fractions by ionizing acidic compounds using basic solution partitioning. The two fractions were purified separately using conventional column chromatography and analyzed by ECNI-GC-MS. Plasma lipids were determined using colorimetric detection. Comparisons to conventional gravimetric lipid determination methods are made herein. The extraction method and lipid determination method were then applied to 71 polar bear plasma samples for method validation.

3.2. Standards, Chemicals and Supplies

3.2.1. Standards

A standard quantitation mixture of OH-PCBs and other halogenated phenolic compounds was prepared for the analysis of polar bear plasma samples. Some standards were provided courtesy of Åke Bergman (University of Stockholm, Sweden) while others were purchased commercially. More standards became available before the experiment was completed, therefore, a second mixture was prepared and samples were re-injected and analyzed for additional compounds. Table 3.1 lists standards, sources and concentrations of Mix 1 and Mix 2. Standards are shown with the functional groups as they were acquired. All compounds were derivatized with diazomethane prior to making the standard quantitation solution. Diluting the stock solution to 25-100 pg/μl for Mix 1 and to 100 pg/μl for Mix 2 established the quantitation solutions.

A previously characterized secondary polar bear quantitation standard (PBQ) was used to quantitate CHCs in the neutral fraction (235). The PBQ was prepared from 150 g of polar bear adipose tissue that was extracted and cleaned up using multiple techniques as described by Norstrom *et al.* (235). All compounds in the extract were then identified or characterized by full scan GC-EI-MS. Compounds were also quantitated using GC-flame ionization detection. Since molar carbon response is relatively constant among compound classes (236), unknown compounds could be quantitated using response factors from structurally similar compounds and knowledge of molecular formulae. The extract was diluted 20 fold for use as a standard and sealed in ampoules (1 ml aliquots) for quantitative use. The concentration of the contaminants found in the PBQ standard ranged from 10-3222 pg/μl. The compounds are shown in Table 3.2 along with ions that were monitored for their detection in the developed SIM method. As seen in Table 3.2, some compound identities are not known. The compounds known as U2, U4, and C5 are chlordane related compounds whose structures have not been determined (64). MC compounds are chlordane components with known structures. Recent work by Karlsson (66) has further identified many of the chlordane compounds and cross-referenced the MC designations with names used in earlier studies.

Table 3.1 - Mixtures of standards used in the quantitation of the halogenated phenolic compounds. Sources of individual standards are indicated. Accustandard (New Haven, CT, USA), Dr. Ehrenstorfer Standards (Atlanta, GA, USA), Stockholm University (from Åke Bergman, Wallenberg Laboratories, Sweden), Wellington Laboratories (Guelph, ON, Canada), EPA (Research Triangle Park, NC, USA).

Phenolic Compound - Mixture 1			Phenolic Compound - Mixture 2		
Compound	Source	Concentration (pg/µl)	Compound	Source	Concentration (ng/µl)
Pentachlorophenol	EPA Research Triangle Park	500	Pentachlorophenol	EPA Research Triangle Park	2.00
2,3,4,5-tetrachlorophenol	EPA Research Triangle Park	500	2,3,4,5-tetrachlorophenol	EPA Research Triangle Park	2.00
2,4,6-trichlorophenol	EPA Research Triangle Park	500	2,4,6-trichlorophenol	EPA Research Triangle Park	2.00
4-OH-CB54	Wellington Laboratories	500	Pentabromophenol	Dr. Ehrenstorfer standards	2.00
4'-OH-CB72	Accustandard	494	2,4,6-tribromophenol	Dr. Ehrenstorfer standards	2.00
4'-OH-CB104	Wellington Laboratories	500	2,4-dibromophenol	Dr. Ehrenstorfer standards	2.00
3'-OH-CB85	Wellington Laboratories	500	2,6-dibromophenol	Dr. Ehrenstorfer standards	2.00
4'-OH-CB121	Accustandard	476	4-OH-heptachlorostyrene	Wellington Laboratories	2.00
4'-MeO-CB120	University of Stockholm	501	4'-MeO-CB97	University of Stockholm	2.00
3-OH-CB118	Wellington Laboratories	500	4'-OH-CB50	Accustandard	2.00
4-MeO-CB109	University of Stockholm	250	4'-OH-CB69	Accustandard	2.00
4'-MeO-CB107	University of Stockholm	250	4'-OH-CB86	Accustandard	2.00
4'-OH-CB165	Accustandard	255	4'-OH-CB93	Accustandard	2.00
4-MeO-CB146	University of Stockholm	250	4'-OH-CB106	Accustandard	2.00
3'-OH-CB138	Wellington Laboratories	250	4'-OH-CB72	Accustandard	2.00
4'-MeO-CB130	University of Stockholm	500	4'-OH-CB121	Accustandard	2.00
4-MeO-CB187	University of Stockholm	1000	3-OH-CB118	Wellington Laboratories	2.00
4'-OH-CB159	Accustandard	250	4'-OH-CB165	Accustandard	2.00
3'-OH-CB180	Wellington Laboratories	500	4-MeO-CB187	University of Stockholm	2.00
4'-OH-CB172	Wellington Laboratories	381	4'-OH-CB172	Wellington Laboratories	2.00
4-MeO-CB193	University of Stockholm	500	4-MeO-CB193	University of Stockholm	2.00

Table 3.2 - Compounds, monitoring and qualifying ions and ion windows for the GC-mass spectral analysis of the neutral fraction isolated using the plasma extraction method.

Retention Time Range (minutes)	Compound	Target/ Qualifier	Retention Time Range (minutes)	Compound	Target/ Qualifier
5.0-8.0	TeClBz	216	18.4-19.2	CB-149	360
	^{13}C - TeClBz	224		U-2	443
				CB-118	326
8-9.4	PnClBz	250		^{13}C - CB118	338
	^{13}C - PnClBz	256		*p,p'*-DDD	235, 237
				CB-146	360
9.4-11.8	HxClBz	284			
	^{13}C - HxClBz	290	19.2-19.9	CB-153	360
	α-HCH	219, 183		^{13}C - CB153	372
	β-HCH	219, 183		CB-105	326
11.8-14.5	Compound C	238	19.9-21.0	*p,p'*-DDT	235, 237
	^{13}C - CB28	268		CB-137	360
	CB-52	292		CB-138/163	360
	^{13}C - CB52	304		^{13}C - CB138	372
	CB-47/48	292		CB-187	394
14.5-16.0	photoheptachlor	339	21.0-25.1	CB-183	394
	OCS	308, 378		CB-156	360
	heptachlor epoxide	353		CB-157	360
	CB-74	292		CB-180	394
	oxychlorodane	387		^{13}C - CB180	406
	U-4	255, 291		CB-170/190	394
				CB-195	430
16.0-17.1	MC2	339			
	CB-56/60	292	25.1-27.9	CB-194	430
	C-5	373, 375		^{13}C - CB-194	442
	CB-99	326		CB-206	464
	MC5	373, 375			
	MC6	409	27.9-31.0	CB-209	498
	t-nonachlor	409			
17.1-18.4	*p,p'*-DDE	246			
	CB-85	326			
	Dieldrin	380			

The following $^{13}C_{12}$ labeled standards acquired from Wellington Laboratories (Guelph, ON, Canada) were used as recovery standards for OH-PCB determination:

4′-OH-CB120, 4′-OH-CB159, 4′-OH-CB172, and 4-OH-CB187. PCP ($^{13}C_6$) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA) and used as the PCP recovery internal standard. Labeled PCBs ($^{13}C_{12}$ - CB28, 52, 118, 153, 180 and 194) were used as recovery standards and $^{13}C_{12}$-CB138 as the performance standard for PCB analysis by the external standard method. The $^{13}C_{12}$-PCB standards were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

Lipid Lin-Trol standards (prediluted set) were purchased from Sigma (Milwaukee, WI, USA) and used as calibration standards for the colorimetric detection of plasma lipids. Standards consisted of 7 different concentrations of triglycerides, cholesterol and HDL cholesterol in a protein based solution.

3.2.2. Chemicals and Supplies

Polar bear plasma samples were collected from 71 polar bears from Hopen and Edgeøya Islands, southeast Svalbard Svalbard, Norway and Lancaster Sound, Nunavut Territory, Canada (near Resolute Bay). Polar bear sampling details are thoroughly described in Chapter 5.

All solvents were residue analysis grade and purchased from EM Science (Gibbstown, NJ, USA). Florisil (Pesticide Analysis Residue grade, 60-100 mesh) was purchased from BDH Inc. (Toronto, ON, Canada). Florisil was activated by heating at 600°C overnight, cooled to 100°C and deactivated with 1.2% (w/w) doubly distilled and deionized water and stored in a capped (Teflon lined) glass bottle. Merck Silica gel (Grade 60, 70-230 mesh, 60Å) was purchased from Aldrich Chemical Company,

Inc. (Milwaukee, WI, USA). Silica was activated by heating for three hours at 180°C and kept in a 100°C oven until just prior to use. Sulfuric acid (Trace metal grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Hydrochloric acid (concentrated 37%) was purchased from BDH laboratory supplies (Poole, England).

Vanillin (4-hydroxy-3-methoxybenzaldehyde) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Phosphoric acid (orthophosphoric acid, 85%) was purchased from Fisher Scientific (Pittsburgh, PA, USA). The vanillin solution was prepared by dissolving vanillin (6.0 g) into water (1 L). A phospho-vanillin reagent (PVR) was made by diluting the vanillin solution (350 ml) with water (50 ml) and concentrated phosphoric acid (600 ml). Both solutions were stable for up to two months in brown bottles at room temperature.

3.3. Instrumental Conditions

Procedures for derivatization and GC-ECNI-MS determination of halogenated phenolic compounds were described in Chapter 2. Neutral compounds (PCBs, chlordanes, chlorobenzenes, DDT and metabolites) were analyzed on a Hewlett Packard (Atlanta, GA, USA) 5890A Series II gas chromatograph equipped with an HP 7673A automatic injector and a Hewlett Packard 5988A mass spectrometer. Helium was used as the carrier gas and head pressure was set at 80 kPa. All injections (2 µl) were made in splitless mode onto a DB-5 ([5%-phenyl]-methylpolysiloxane - 30m x 0.25mm i.d., 0.25 µm film thickness; J&W Scientific Inc., Folsom, CA, USA.) column. Injector temperature was set at 250°C. The electron energy was 70 eV and the source temperature was 200°C. The GC temperature

program for the neutral compound analysis was 100°C for 3 minutes, 10°C/min to 180°C and then 2.5°C/min to 280°C.

Lipids were determined colorimetrically by reacting with PVR and measuring absorbance for each sample at 540 nm on a Hewlett-Packard Diode Array Spectrophotometer HP8452A (Palo Alto, CA).

3.4. Results and Discussion

3.4.1. Methodology

Numerous methods, including mixing with sodium sulfate followed by solvent extraction, and solid phase extraction, were tested for the extraction of halogenated phenolic compounds from plasma. The former method was inefficient and solvent intensive while the latter provided inconsistent results. Therefore, more conventional and consistent methods involving liquid-liquid extraction were chosen.

3.5. Extraction Procedure

The method of extraction for both whole blood and plasma (Figure 2.2) was a modification of the Wallenberg Plasma Extraction (WPE) method (5, 220). Samples were removed from the freezer and allowed to thaw. Plasma samples (mass range 0.91 to 4.31 g) were weighed into glass stoppered centrifuge tubes (50 ml) and spiked with $^{13}C_{12}$ recovery standards prior to extraction. The standards consisted of a PCB/chlorobenzene mixture (10 µl: $^{13}C_{12}$ - CB-28, 52, 118, 153, 180 and 194, 2.5 ng/µl, $^{13}C_6$ – TeClBz, PnClBz and HCB, 2.0 ng/µl), a OH-PCB mixture (20 µl: $^{13}C_{12}$ - 4′-OH-CB120, 4′-OH-CB159, 4′-OH-CB172 and 4-OH-CB187, 1.00 ng/µl) and PCP ($^{13}C_6$ labeled – 20 µl, 100 pg/µl).

Samples were vortexed to mix and allowed to equilibrate for 20 minutes. Plasma proteins were denatured by the addition of hydrochloric acid (6 M, 1 ml) and 2-propanol (3 ml). The samples were then vortexed again briefly to mix. Addition of methyl-*tert*-butyl ether (MTBE):hexane (1:1, 6 ml) was followed by a one minute vortex and 30 minute centrifugation (Sorvall Angle Centrifuge, Model NSE). The white protein precipitate gathered in the bottom aqueous phase and the top organic phase was transferred to a separatory funnel (125 ml). The extraction using MTBE:hexane (1:1, 6 ml) was repeated two more times and the organic phase were added to the separatory funnel each time. Samples were then treated with potassium chloride (1%, 6 ml) and shaken for 1 minute. Phases were allowed to separate and the bottom aqueous phase, also containing the co-extracted 2-propanol, was drained and discarded. The remaining organic phase was drained into flat bottom flasks (125 ml) and roto-evaporated until approximately 1 ml in volume. The 1 ml extract was transferred to a separatory funnel (125 ml) with three washes of hexanes (1 ml) and partitioned with potassium hydroxide (1 M in 50% ethanol, 6 ml). The separatory funnels were shaken for 1 minute and the bottom aqueous phase containing the ionized phenolic compounds was drained into a screw top centrifuge tube (50 ml). Partitioning was repeated two more times and the organic phases added to the previous wash. The collected aqueous phases (Fraction 1) were then re-washed with hexanes (3 ml) two times to extract any co-extracted PCBs and other CHCs present in the aqueous phase. The hexane washes were added to the organic phase (neutral fraction) from the partitioning step (Fraction 2).

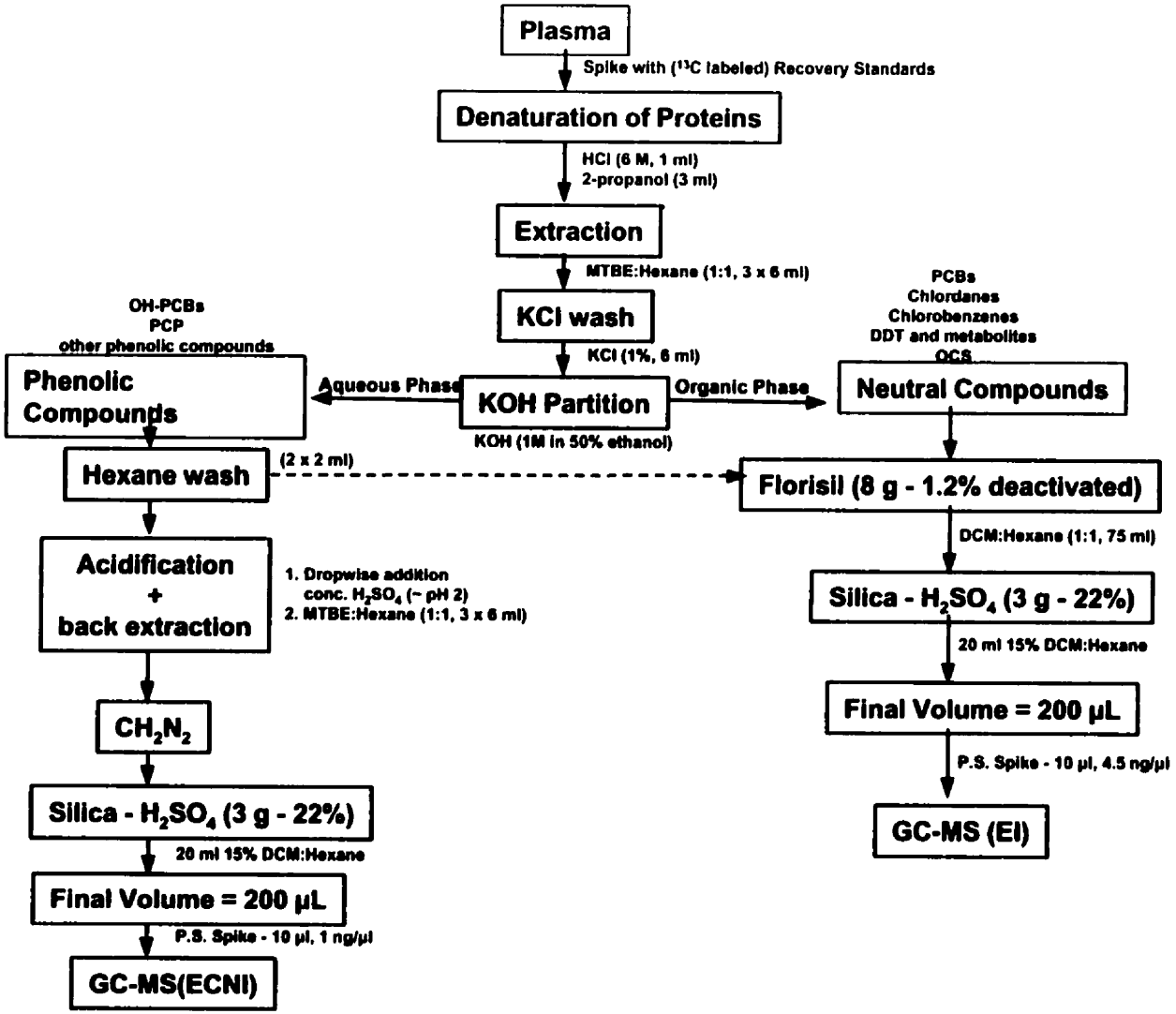


Figure 3.1 - Flow chart showing the extraction and clean up procedure for the determination of phenolic and neutral compounds from plasma and whole blood.

Fraction 2 was roto-evaporated to less than 1 ml and cleaned up on Florisil (8 g, 1.2% deactivated with distilled and deionized water). The CHCs were eluted with dichloromethane (DCM):hexane (1:1, 75 ml) and roto-evaporated to 1 ml volume. This was applied to a silica-sulfuric acid column (22% H_2SO_4, 3 g) and eluted with 15% DCM:hexane. (20 ml). The samples were brought down to final volume (200 µl) and spiked with $^{13}C_{12}$ labeled CB138 performance standard (4.5 ng/µl, 10 µl).

Fraction 1 was acidified with concentrated sulfuric acid (dropwise addition until pH 2) to protonate phenolic anions and extracted with MTBE:Hexane (1:1, 6 ml). The sample was vortexed to mix and phases were allowed to separate (some samples required centrifugation to lower the white precipitate below interface level). The top organic phase containing phenolic compounds was transferred to a column containing a small plug (3 cm) of sodium sulfate to further dry the extract and drained into a flat bottom flask (125 ml). Back extraction was repeated two more times and applied to the same sodium sulfate column. The collected organic fractions (containing acidic compounds) were roto-evaporated to less than 1 ml and derivatized with diazomethane to give methyl ether derivatives of phenolic compounds. The solution remained yellow after derivatization indicating excess diazomethane. The solution was roto-evaporated to less than 1 ml. The methyl ethers were then further purified by applying them to a silica-sulfuric acid column (22% H_2SO_4, 5 g) to remove residual biogenics and eluted with DCM:hexane. (15% DCM, 40 ml). Fraction 1 was reduced to final volume (200 µl) and spiked with performance standard - 4′-Me-4-MeO-2,3,3′,5,6-pentachlorobiphenyl (4′-Me-4-MeO-CB112 - 20 µl, 200 pg/µl).

3.6. Lipid Determination

Gravimetric techniques of plasma lipid determination and a colorimetric technique were compared to determine the most efficient method of analysis.

Gravimetric methods are advantageous in that they generally use part or all of the extract used in the chemical analysis prior to column chromatography purification, thus not requiring much extra work to prepare a sample for lipid determination. However, when part of the extract is used, there is a loss of sample that may effect detection of certain compounds. When all the extract is used, the sample is evaporated down to dryness, possibly resulting in loss of volatile compounds. First, the current method of phenolic compound extraction was tested for extraction efficiency of lipids as compared to the classic Sperry and Brand (S&B) method (237). Next, a colorimetric method was considered as an alternative to the conventional gravimetric determination. In addition, methanol was tested to demonstrate its efficacy as a deproteination agent prior to lipid extraction.

All lipid determination methods were compared on pooled samples of polar bear plasma. Separate 4 ml aliquots were taken and frozen at –20°C.

3.7. Gravimetric Determination

Plasma samples were thawed, dispensed in aliquots (1.00 g) and extracted according to the phenolic compound extraction (PCE) method described above and by the S&B method (237) as modified by Jacobs and Henry (238). The PCE method involved addition of HCl (6.0 M, 200 µl), 2-propanol (600 µl), and MTBE:Hexane

(1:1, 2 ml). The mixture was vortexed and the phases allowed to separate. The top organic phase was transferred into a pre-weighed aluminum dish. The MTBE:Hexane addition was repeated two more times and added to the aluminum dish. The dish was then placed in a fume hood, allowed to evaporate to dryness and weighed on an analytical balance (5 digit, Sartorius, AG model BP21OD, Goettingen, Germany).

Since many plasma extraction methods use methanol as a deproteination agent, the effect of methanol on efficiency for extracting lipids was tested. Using a similar procedure to the one described above, different extracting solvents (2 ml) were used along with varying amounts of methanol (% based on plasma volume where 1 g = 1 ml). For example, 28% methanol would indicate 280 µl of methanol per 1 g of plasma. Briefly, methanol was added to dispensed aliquots of plasma (1.00 g) followed by the addition of extracting solvent (2 ml – hexane, hexane:DCM or hexane:MTBE) and vortexed to mix. The top organic layer was removed and the extraction repeated two more times. The combined organic phases were transferred to preweighed aluminum dishes and weighed as described above.

The S&B method was a much more exhaustive method (237). Plasma (1.00 g) was placed in a volumetric flask (50 ml) and mixed with methanol (16 ml). The sample was then diluted to the mark with chloroform, mixed thoroughly, filtered through filter paper (Whatman 1) and 40 of the 50 ml was transferred to a beaker (100 ml). Water (distilled and deionized) was added to within 1 cm of the top of the beaker. The small beaker (100 ml) was carefully placed into a larger beaker (1000 ml), which was filled to within 3 cm of the top. The little beaker, immersed in water, was allowed to equilibrate and sit overnight. The water equilibration allows the

purification of the lipids by the removal of co-extracted water soluble contaminants from the lipids, such as sodium chloride, urea, and uric acid (238). Water was aspirated off, leaving a small amount on the chloroform phase. The chloroform and residual water were evaporated to dryness on a steam bath with the aid of a gentle stream of nitrogen. The residue was dissolved with warm chloroform:methanol (2:1, 2 ml) and filtered through a sintered glass filter under vacuum. The filter was washed four times with chloroform:methanol (2:1, 2 ml). The filtrate was reduced in volume with a gentle stream of nitrogen and transferred to a preweighed aluminum dish. The remaining solvent was evaporated in a fume hood and weighed on an analytical balance to determine lipid percentage and applying a correction factor for using 40 of the 50 ml as described above.

Each gravimetric technique was repeated five times for intra-method comparisons.

3.8. Colorimetric Determination

The colorimetric method of analysis provides an alternative method of determining lipids. They generally require small volumes of plasma and are therefore useful when sample sizes are limited.

Chabrol and Charonnat originally developed colorimetric detection of total lipids in 1937 (239). The method has been modified by Frings *et al.* (240, 241). Briefly, Lin-Trol standards, water blank and plasma samples (20 µl) were transferred to a centrifuge tube (15 ml, ground glass stopper). Concentrated sulfuric acid (200 µl) was added and each tube vortexed to mix. The tubes were reacted in a water bath for

10 minutes at 100°C, removed and cooled in ice cold water for 5 minutes. PVR (10 ml) was added to the black samples and vortexed. The samples were allowed to react for 15 minutes at 37°C in a water bath and cooled in ice water for an additional 5 minutes. A small aliquot of each purple colored solution was transferred to a cuvette prior to measuring the absorbance at 540 nm. The reaction products deteriorated with time thus all samples were measured within 30 minutes of PVR addition.

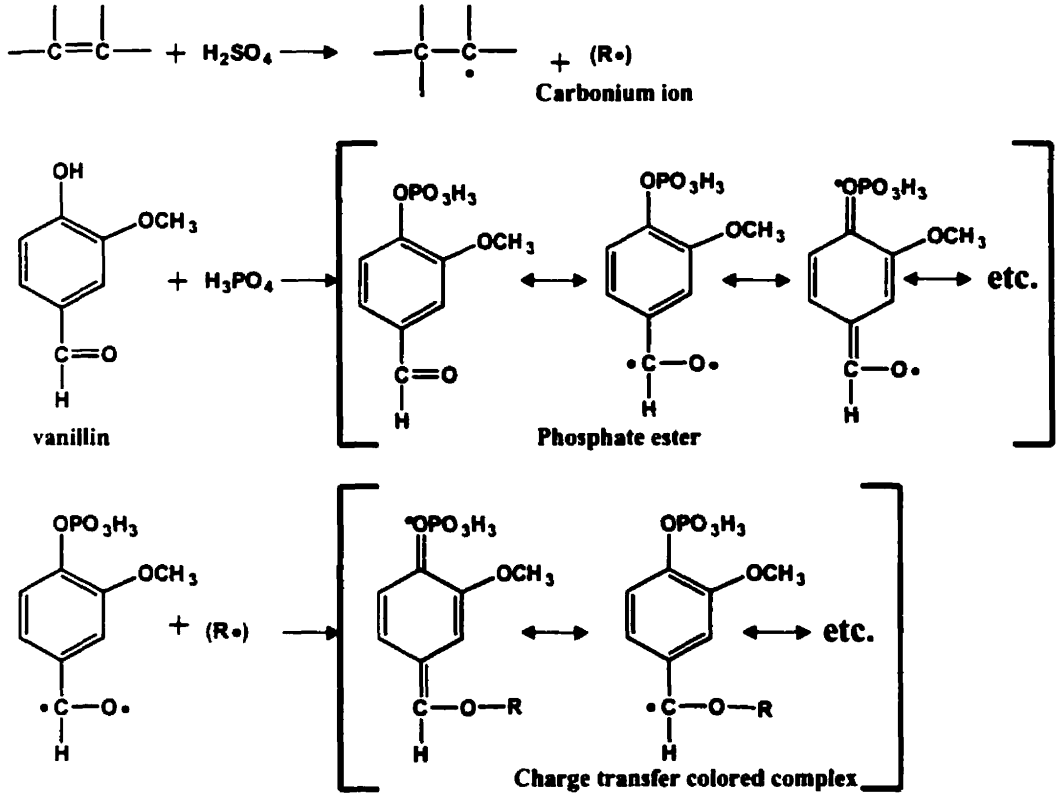


Figure 3.2 - Proposed reactions for plasma lipids and the vanillin reagent in the colorimetric determination of lipids.

The reaction sequence to form the colored products is proposed to proceed through three steps as shown in Figure 3.2 and described by Knight *et al* (242). The

first step involves the formation of carbonium ions from unsaturated bonds in lipid fatty acids. The second step involves the formation of aromatic phosphate ester from phosphoric acid and vanillin. The final step is a reaction between the activated carbonyl group of phopho-vanillin and the carbonium ion, which forms a colored charge-transfer complex, stabilized by multiple resonance structures. Five replicates of polar bear plasma pool were completed for comparison to the gravimetric techniques.

3.9. Method Validation

Polar bear plasma (n=71) was used in the validation of the extraction and quantitation method for OH-PCBs and other halogenated phenolic compounds and the determination of plasma lipids. Plasma samples were extracted and cleaned up as described above, and analyzed according to the procedures in Chapter 2.

3.10. Results and Discussion

The quantitative determination of CHC contaminants in tissues, including plasma, frequently requires accuracy in the pg range and precision within ± 10% CV. If plasma concentrations are lipid normalized, additional uncertainty is added due to the lack of consistency in results from various lipid determination techniques employed by different laboratories. Many laboratories use solvent extraction and gravimetric analysis of lipids. Choice of solvent is usually dictated by optimum recovery of CHCs with minimal co-extraction of lipids. In tissues where non-polar, neutral triglycerides dominate the lipid fraction, such as fat and liver, commonly

employed extraction solvents have similar extraction efficiency for lipids. Lipid and lipophilic components of plasma include cholesterol and cholesterol esters, phospholipids, lipoproteins and triglycerides. These are much more variable in their polarity and therefore extractability by various solvents, resulting in higher or lower values depending on the solvents used. This can make lipid results difficult to interpret and it is almost impossible compare lipid normalized data generated by different laboratories. Since the lipid percentage in plasma is small to begin with, an error in such a small number can change the lipid normalized contaminant concentrations dramatically.

To demonstrate the effect of the deproteinization agent and extracting solvent on extractable lipid components, different solvents and different amounts of deproteinizing agent (methanol) were compared to the reference lipid analysis method (S&B). As shown in Figure 3.2, the amount of methanol and choice of solvent was critical to ensure comparable lipid recovery to the S&B method. One volume of methanol to one volume of plasma was needed to extract similar amounts of lipid components using Hex:MTBE as with the S&B method. The PCE method gave ca. 20% higher results than the S&B method. Thus, the PCE method is efficient at extracting total lipids as well as CHCs, and can be used for gravimetric determination of lipids, assuming adequate sample size is available.

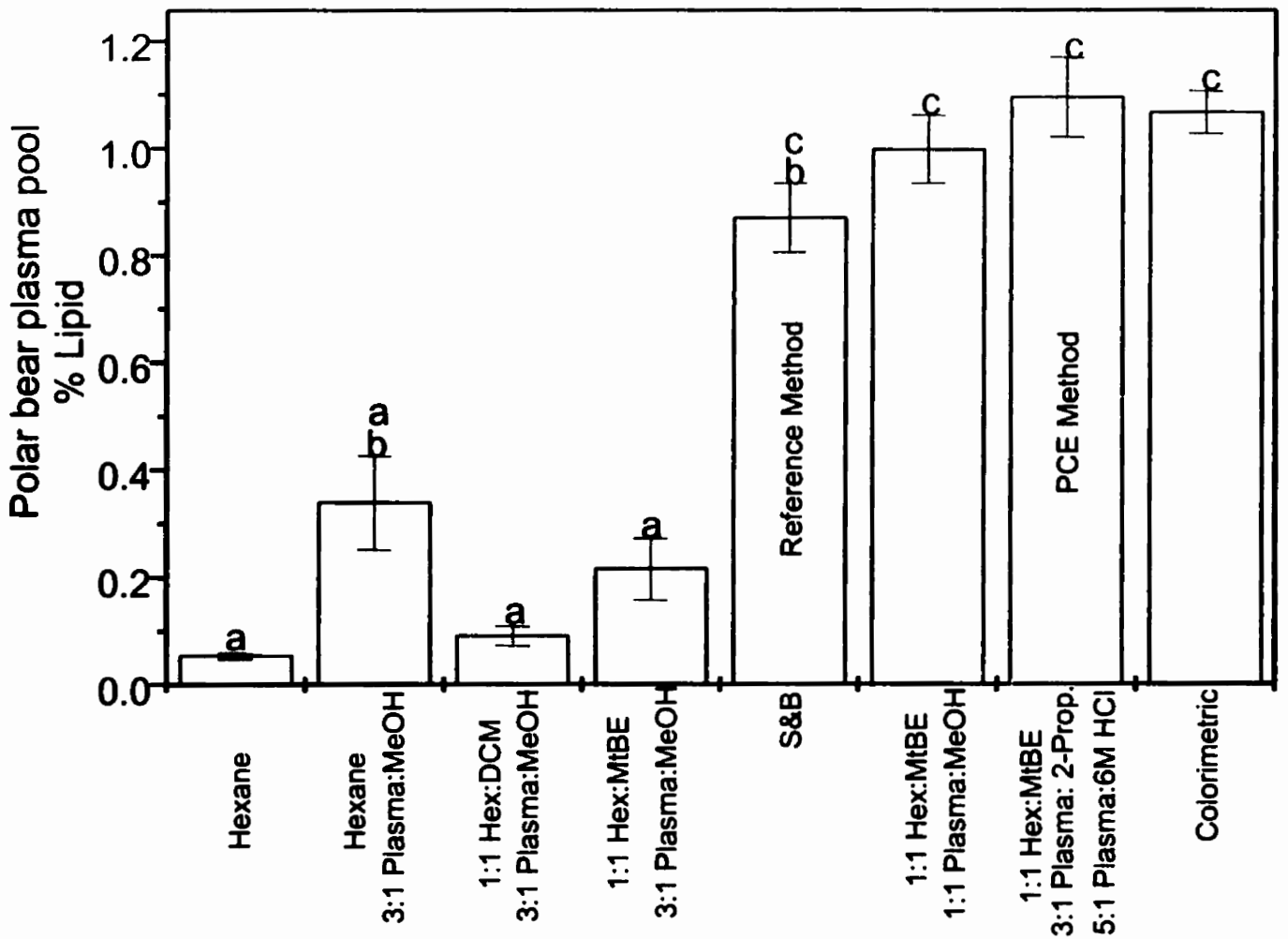


Figure 3.3 - Method comparison of gravimetric techniques showing the effect of methanol on extraction efficiency of lipid components. Sperry & Brand (S&B) is a standard method which is shown along with the colorimetric method (MeOH = methanol, 2-prop = 2-propanol, hex= hexane, DCM = dichloromethane, MtBE = methyl*tert*butyl ether). Extraction solvent was 2 mL per 1 g plasma for each method except S&B.

The colorimetric technique gave similar lipid values to the PCE method, i.e., slightly higher than the reference method. Values were not significantly different (ANOVA) among any of optimized methods (Figure 3.2). The colorimetric method requires a double bond to be present for the formation of the reactive carbonium ion. The colorimetric method was rapid, required small amounts of plasma and gave similar values to the gravimetric methods tested. Therefore, it was applied to the analysis of 71 polar bear plasma samples by reserving 20 µl of plasma from the original plasma extraction. The mean lipid percentage in the polar bear samples was 0.61% ± 0.17% (SD) (range 0.28-1.1%).

Recoveries of ^{13}C-labeled recovery standards are shown in Table 3.2 along with the standard error for all samples. The recovery of ^{13}C labeled standards from polar bear plasma was good, >80% for all internal standards. Recoveries of the labeled OH-PCBs and PCP can be greater than 100% because there is some experimental error in the derivatization and in reducing them to final volume. For example, if the nitrogen blow down step is more vigorous for the standard recovery mixture as compared to a sample, more analytes in the recovery standard may be lost by volatilization. When the sample is compared to this standard, the resulting percentage recovery may be fictitiously higher. Thus, it is important that this step of the procedure is monitored sufficiently to reduce this type of discrepancy between samples and the recovery standard.

Table 3.3 - Recoveries and standard errors (SE) of all the internal standards from polar bear plasma (n=71).

Compound	Mean % Recovery	SE
Neutral Compounds		
TeClBz	81.2	2.1
PnClBz	91.6	1.9
HCB	80.4	1.5
CB28	99.5	1.7
CB52	94.5	1.6
CB118	92.2	1.5
CB153	86.1	1.3
CB180	100.5	2.2
CB194	88.8	4.0
Phenolic Compounds		
PCP	97.5	3.4
4'-OH-CB120	87.5	2.0
4'-OH-CB159	101.3	3.2
4'-OH-CB172	107.0	3.8
4-OH-CB187	106.8	5.3

Lipid normalization is commonly applied to hydrophobic contaminant data for easy comparison to other tissues since many contaminants in blood are associated with the plasma lipid/lipoprotein fraction (243, 244). Phenolic compounds are thought to be mostly associated with the thyroid hormone transport proteins (102) and may not be strongly associated with the lipid/lipoprotein fraction. As explained by Hebert and Keenleyside (245), lipid normalization is needed only when lipids are introducing a bias to the data set. If lipids are not correlated with contaminants, there is no need for lipid normalization of the data. Therefore, chemical classes (chlorobenzenes, HCHs, chlordanes, PCBs and OH-PCBs) were tested for a relationship with plasma lipids in the 71 polar bear plasma samples. No significant relationship was determined for any

of the chemical classes (Table 3.4). Therefore, the data was not lipid normalized prior to further statistical analysis (Chapter 5).

Table 3.4 – Correlation coefficients between plasma lipids and sum compound classes. No relationships were statistically significant ($p>0.05$).

Compound Class	Correlation Coefficient
Sum ClBzs	0.02
Sum HCHs	0.16
Sum DDT	0.07
Sum Chlordanes	0.18
Sum PCBs	0.12
Sum OH-PCBs	0.06

In conclusion, a reliable method for extraction and quantitation of both neutral CHCs and acidic compounds (OH-PCBs and other halogenated phenolic compounds) was successfully developed and tested on 71 polar bear plasma samples. Various attempts at simplifying the extraction method using solid-phase extraction were unsuccessful due to poor and erratic recoveries of OH-PCBs. Although time-consuming, the liquid/liquid method was simple and effective in the extraction of the phenolic compounds as well as the neutral compounds found in the polar bear plasma. Recoveries of standards were very consistently good (80-100%) and precision was also very good (SE 1.5 – 5.3%), indicating that the method is successful at extracting all the desired analytes. A colorimetric lipid determination method was found to be as accurate as standard gravimetric techniques. Colorimetric determination of plasma lipids was fast and efficient without using significant amounts of sample or sample extract.

3.11. Acknowledgements

The author acknowledges Environment Canada and the National Wildlife Research Center for their support of this research. Lipid determination experiments were carried out in collaboration with Ken Drouillard (Trent University, Peterborough, ON, Canada) and presented as a poster at the SETAC conference in 1998.

Chapter 4. Identification of 4-hydroxyheptachlorostyrene (4-OH-HpCS) in polar bear plasma and its binding affinity to transthyretin (TTR): a metabolite of octachlorostyrene (OCS)? †

4.1. Background

Chlorinated hydrocarbon (CHCs) pesticides and industrial chemicals have been studied extensively due to their ubiquitous presence in biota. Phenolic metabolites of CHCs isolated from plasma have received particular interest (5). Phenolic compounds are capable of modulating hormone-mediated processes *in vitro* and *in vivo*, via processes dependent on the estrogen receptor (176) and the disruption of thyroid hormone (246) and vitamin A (retinol) transport (102). For example, hydroxylated polychlorinated biphenyls (OH-PCBs), phenolic metabolites retained in plasma of wildlife and humans (151, 231), have demonstrated potential estrogenic activity (190) and thyroidogenic effects *in vitro* and *in vivo* (136).

The prohormone thyroxine (T4) is transported to target tissues by thyroid hormone transport proteins, such as transthyretin (TTR). The deiodinase family of enzymes present in these target tissues converts T4 to the active hormone, triiodothyronine (T3) (247). Aromatic compounds that have OH-groups with adjacent halogen substituents can bind with high affinity to TTR (136). The binding potency of pentachlorophenol (PCP) and 4′-OH-3,3′,4,5′-tetrachlorobiphenyl toward TTR was 2 and 4 times greater, respectively, than the natural hormone, T4 (72, 142).

---

† Adapted from C.D. Sandau, I.A.T.M. Meerts, R.J. Letcher, A. McAlees, B. Chittim, A. Brouwer, and R.J. Norstrom, **2000**. *Environ. Sci. Technol.* 34(18):3871-3877.

Hydroxylated polybrominated diphenyl ethers (OH-PBDEs) with T3- and T4-like structures (i.e., bromine atoms adjacent to the phenolic OH-group) have been reported to bind to the human α- and β-thyroid hormone receptor (248), and compete with T4 for binding with TTR *in vitro* (145).

Biotransformation has been shown to generate other phenolic compounds that may be capable of binding to TTR. In rats, hexachlorobenzene (HCB) is biotransformed *in vivo* to PCP (70) and octachlorostyrene (OCS) is converted to a heptachloro metabolite and a pentachlorophenyldichloroacetic acid metabolite (249). The presence of a heptachloro metabolite suggests that the pathway for biotransformation of OCS may be via dechlorination and possibly hydroxylation.

Polar bears are the top predator in the arctic marine food chain, eating primarily ringed seal. Consequently, they bioaccumulate high concentrations of several CHCs, most notably PCBs and methylsulfone PCB metabolites (235, 250, 251). Polar bears are an important biomonitoring species of CHC exposure in the arctic (235). The high body burden of contaminants can induce enzymes of the hepatic cytochrome P450 system, which is responsible for the metabolism of both endogenous compounds and xenobiotics (252). The polar bear has demonstrated a high capability to metabolize several CHCs, including 4,4′-DDE and many PCB congeners (230). It is reasonable to suppose that persistent OH-PCB metabolites would be formed in polar bear, as is the case for other mammals (231) and humans (151). A study was initiated to examine the presence and identity of OH-PCBs in polar bear plasma and their possible effects on circulating retinol and thyroid hormone levels. The results of this study are discussed in Chapter 5.

During methodological development for the extraction and quantification of OH-PCBs, an unknown phenolic compound was detected in polar bear plasma (203). The unknown compound was frequently the major peak in the methylated phenolic compound fraction chromatogram.

We report on the identification of the unknown phenolic compound in polar bear plasma, which we hypothesized to be a hydroxylated metabolite of OCS based on preliminary mass spectral evidence. Identification involved the bulk extraction of polar bear plasma and purification of the unknown compound for mass spectral analysis using high and low-resolution mass spectrometry. The hypothesized compound was then synthesized. Comparisons of the mass spectra and gas chromatographic retention times on three GC columns with varying polarities were used to determine if the synthesized compound was the hypothesized phenolic OCS metabolite. In an attempt to resolve the significance of biotransformation as a source of this phenolic compound in the polar bear, its concentration and the concentration of OH-PCBs, OCS and PCBs were determined in polar bear and ringed seal plasma. OCS and PCBs in polar bear adipose tissue and liver were also quantitated. The presence of TTR in polar bear plasma was determined by polyacrylamide gel electrophoresis (PAGE) separation of proteins and protein binding of ^{125}I-labeled T4. High binding affinity to TTR is the presumed mechanism of selective retention of phenolic compounds in plasma (136). A competitive binding assay using purified human TTR and ^{125}I-labeled T4 was used to study the TTR binding affinity of the phenolic OCS metabolite relative to the natural thyroid hormone, T4.

4.2. Samples

Polar bear plasma samples used in this study were collected from 30 polar bears captured between April and May 1997 by the late Malcolm Ramsay (University of Saskatchewan, Saskatoon, SA) around Resolute Bay (Nunavut Territory, Canada). Whole blood was drawn into 50 ml heparinized vacutainers and stored on ice and out of light until processed. Blood samples were centrifuged and the plasma drawn off, frozen at – 40°C and stored until further analysis.

Plasma samples (n = 5) from ringed seal (age and gender unknown), the main prey species of the polar bear, were similarly collected in 1999 from around Kuujjuaq, Quebec by Inuit hunters (Anguvigaq Hunting, Trapping and Fishing Association, Kuujjuaq, Quebec) and organized by Daniel Leclair (Nunavik Research Centre, Makivik Corporation, Kuujjuaq, Quebec). The plasma samples were stored frozen at -40°C until analysis.

Polar bear adipose tissue and liver samples (n = 8) were collected by the late Malcolm Ramsay (University of Saskatchewan, Saskatoon, SA) in legally controlled hunts by Inuit in the Canadian Arctic during the spring of 1992, 1993, and 1994 near Resolute Bay, Nunavut Territory. Details of the sampling and sample preparation are given by Letcher *et al.* (230, 252).

4.3. Isolation and Mass Spectral Characterization of the Unknown Chlorinated Phenolic Compound

To obtain clean fullscan spectra, a large pooled sample of polar bear plasma (~65 g) from three bears was prepared. Plasma was extracted and the phenolic

compounds were partitioned into potassium hydroxide, acidified and back-extracted using methodology described in Chapter 3. The OH-containing compounds were methylated to their corresponding methoxy-derivatives by treatment with diazomethane (221). After further clean up steps, which included gel permeation chromatography, multiple sulfuric acid partitioning (220), silica:sulfuric acid column treatment (151) and Florisil column chromatography (253), the methylated unknown compound was isolated by applying the methylated fraction to a Florisil column (8.0 g, 1.2 % deactivated) and eluting with hexanes. Fractions of 5 ml were collected and analyzed for the presence of the unknown compound using GC with electron capture detection (ECD). GC-ECD was performed on a Hewlett-Packard (Palo Alto, CA, USA) 5890 instrument equipped with a ^{63}Ni ECD detector and HP7673A automatic injector. The GC was fitted with a fused silica DB-5 column ([5%-phenyl]methylpolysiloxane from J&W Scientific Inc., Folsom, CA, USA, 30 m x 0.25 mm i.d., 0.25 μm film thickness). The carrier gas was helium and the ECD makeup gas was 5% methane-95% argon. All injections were 2 μl in volume and made in splitless mode. The GC temperature ramping was the same as that described previously (Chapter 2) for OH-PCBs. The injector port and interface temperatures were 250°C and 280°C, respectively. Fractions from 10 ml to 25 ml contained the majority of the unknown compound. These were pooled and concentrated for analysis using both high and low-resolution fullscan mass spectrometry.

GC-MS (low-resolution fullscan) analysis was performed with a Hewlett-Packard 5988B instrument. For fullscan electron capture negative ionization (ECNI) mode, the GC and MS conditions are described in detail in Chapter 2. Methane

(99.99% pure) was the reagent gas at a source pressure of 2.5 x 10^{-4} torr. The source temperature was 140°C. For EI mode, source temperature was set at 120° and electron energy was set at 70 eV. Fullscan spectra for ECNI and EI modes were obtained from 75 to 650 and 250 to 600 amu, respectively.

High-resolution spectra were obtained with a VG AutoSpec double-focusing mass spectrometer (resolution 10 000) in fullscan EI mode. The instrumental conditions are described in Tittlemier *et al.* (254).

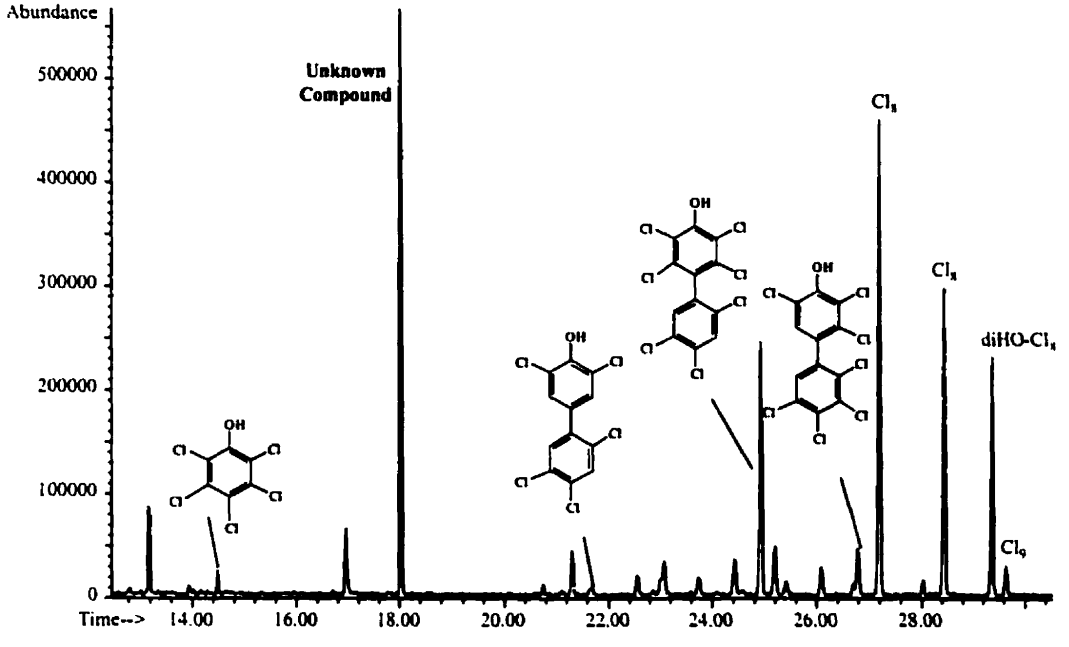


Figure 4.1 - Electron-capture negative ionization mass spectra (fullscan) chromatogram of the phenolic compound fraction from polar bear plasma. The compounds have been methylated but are shown as hydroxylated precursors.

The methylated phenolic compound fraction was initially analyzed using GC/ECNI-MS because of interfering biogenic residue still present following the initial extraction procedure (151). Interferences were minimized using ECNI, since biogenic compounds generally have low electron-capturing capability compared to halogenated

compounds. A GC/ECNI-MS chromatogram of the methylated phenolic compound fraction of a polar bear plasma sample is shown in Figure 4.1. In addition to several OH-PCB congeners, there was a large unknown compound eluting nearly 3.5 minutes after PCP and approximately 3.5 minutes before the first eluting OH-PCB. The retention time indicated that the compound could either be a chlorinated monocyclic aromatic compound with a side chain or a lower chlorinated OH-PCB.

The ECNI spectrum of the methylated unknown phenolic compound is shown in Figure 4.2. The base peak at 322 amu had an isotope pattern indicative of a hexachlorinated species. There was only one detectable isotope cluster at higher mass (338 amu). The isotope cluster was characteristic of a compound with six chlorines and must contain a methoxy group because only methylated phenolic compounds are present in this fraction. The formula best matching this fragment was $[C_9H_4Cl_6O]^-$, suggesting that the unknown compound was most likely a mono-aromatic with a side chain. Although this formula was consistent with a methoxyhexachlorostyrene (MeO-HxCS), loss of $[M-16]^-$ to form the fragment at 322 amu was unlikely. However, addition of $H^\bullet$ to molecular and fragment ions frequently occurs in ECNI-MS (225). Addition of $H^\bullet$ and loss of $CH_3^\bullet$, $[M+H-CH_3]^-$, from $[C_9H_4Cl_6O]^-$ could explain the peak at 322 amu. This does not preclude that the ion at 338 amu was $[M-X+H]^-$, where X may be a halogen or radical fragment. Therefore, MeO-heptachlorostyrene (MeO-HpCS, no molecular ion) or MeO-hexachlorostyrene (MeO-HxCS) isomers were consistent with the ECNI spectrum. No other significant structural information could be inferred from the ECNI spectra except fragment ions corresponding to the successive loss of chlorines.

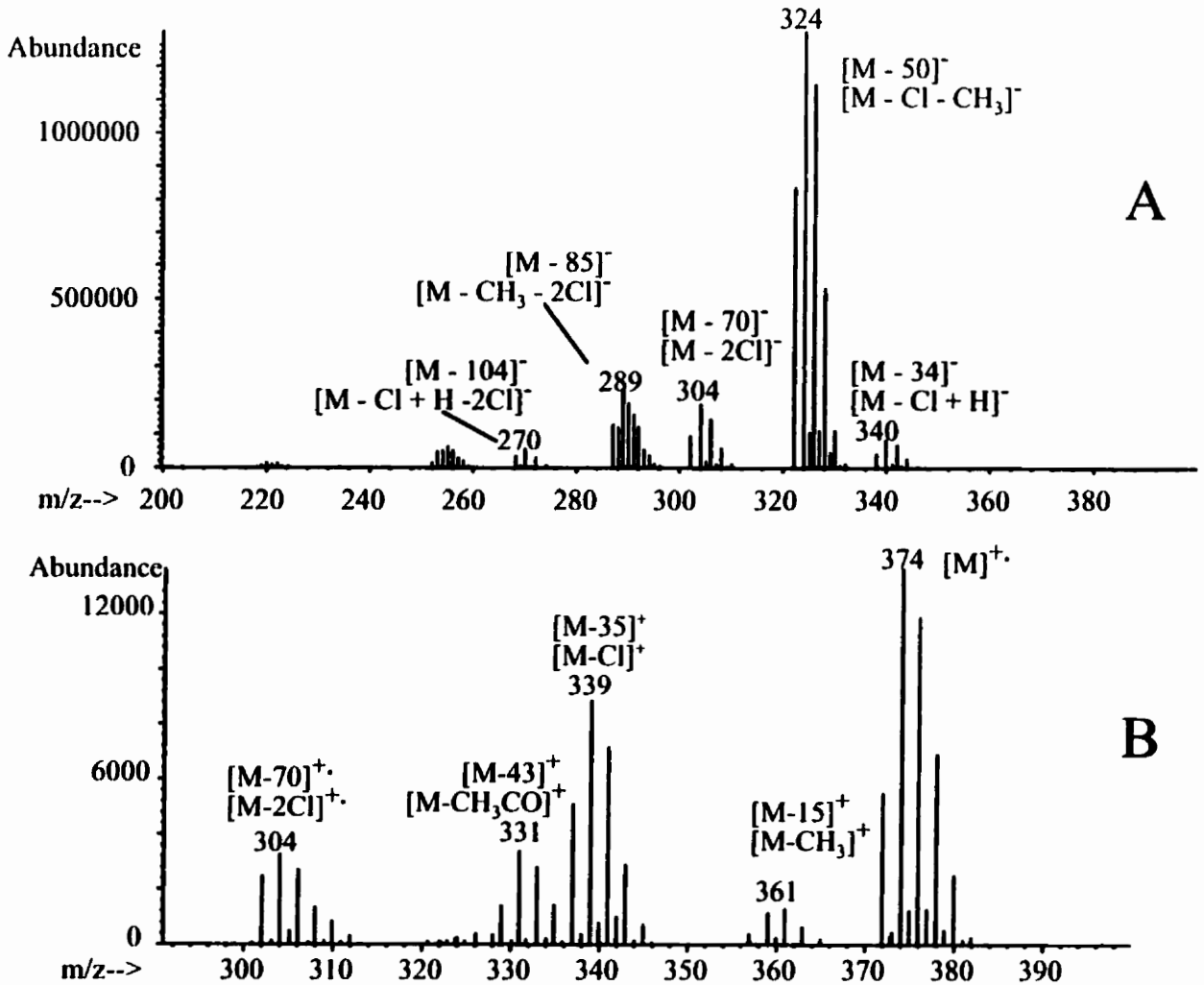


Figure 4.2 - Low resolution (A) ECNI (200-400 amu) and EI (290-400 amu) ionization mass spectra of the unknown methylated phenolic compounds isolated from polar bear plasma (see Figure 4-1).

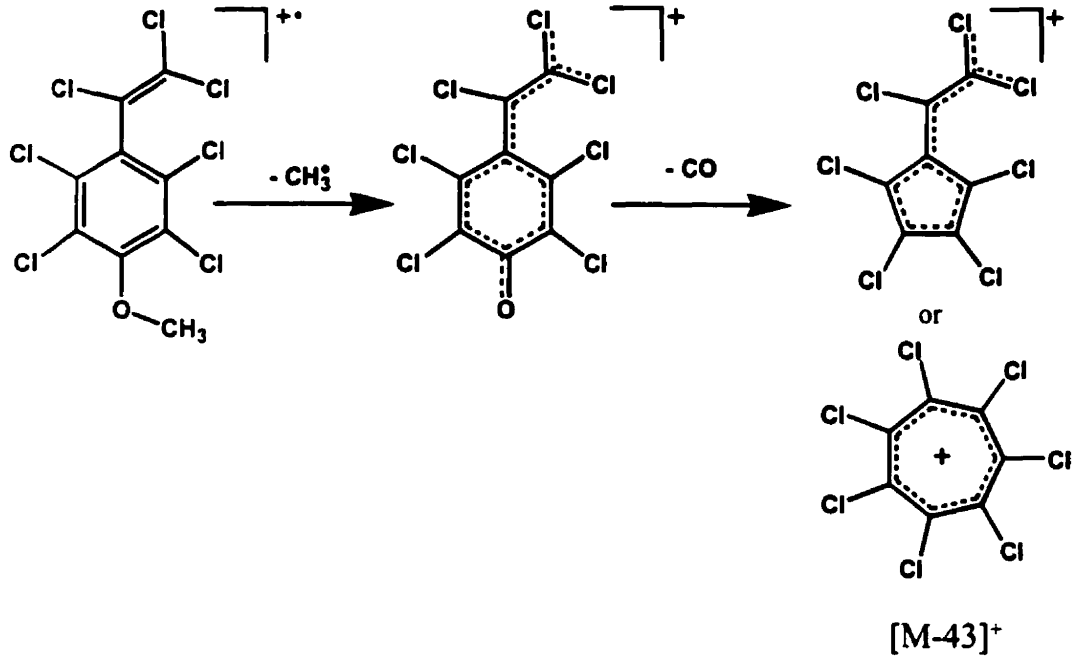


Figure 4.3 - Proposed fragmentation of 4-methoxy-heptachlorostyrene by electron impact mass spectrometry for the formation of $[M-43]^+$ ion.

The molecular ion of the unknown phenolic compound was discovered by EI-MS. The low-resolution EI spectrum indicated that the molecular ion was a heptachlorinated species (Figure 4.2). The $[M]^+$ of 372 amu corresponded to $[C_9H_3Cl_7O]^+$, which was most consistent with a MeO-HpCS isomer. In Figure 4.2, fragments are labeled for loss of mass units and their corresponding structures based on this assignment. The EI spectrum showed distinctive loss of a methyl group, a chlorine and an $[M-43]^+$ ion, which represented an $[M-CH_3CO]^+$ fragment ion. All these fragmentation patterns have been observed previously for MeO-PCBs (211). The $[M-CH_3CO]^+$ fragment is indicative of a para-substituted, aromatic compound when accompanied by an $[M-CH_3]^+$ fragment (255). Fragmentation is thought to

entail a two step process – the loss of a methyl radical followed by the loss of carbon monoxide and subsequent formation of a non-aromatic cyclopentadienyl ring (Figure 4.3). The formation of a $[M-43]^+$ ion was demonstrated previously for polymethoxybiphenyls (256). Para-substitution of the aromatic ring was the only molecular structure that allows for charge delocalization to the ethylene side chain and electronic stabilization of the proposed $[M-CH_3]^+$ fragment ion. The $[M-CH_3CO]^+$ fragment is stabilized by charge delocalization through conjugation of the cyclopentadienyl group to the ethylene side chain. The $[M-CH_3CO]^+$ may also be stabilized by the formation of a heptachlorotropylium cation which has been demonstrated in toxaphene congeners (257) (Figure 4.3).

Table 4.1 - Elemental compositions derived from high resolution EI-MS of the four major ions (see Figure 4.2) of the methylated unknown phenolic compound and the methylated 4-OH-HpCS synthesized standard. The sample and standard were analyzed at a resolution of 10 000.

Fragment	most probable elemental composition	Theoretical Mass	**Sample** Experimental Mass	Difference	**Standard** Experimental Mass	Difference
M^+	$C_9H_3OCl_7$	371.8004	371.7999	0.0005	371.7976	0.0027
$[M-15]^+$	C_8OCl_7	356.7769	356.7802	-0.0033	356.7762	0.0007
$[M-35]^+$	$C_9H_3OCl_6$	336.8315	336.8323	-0.0008	336.8303	0.0012
$[M-43]^+$	C_7Cl_7	328.7833	328.7863	-0.0029	328.7820	0.0014

The elemental composition of the molecular ion and the first four major ion clusters in the EI spectrum (Figure 4.2) were confirmed using high-resolution EI-MS. The most probable elemental compositions were calculated by software provided as part of the OPUS operating system of the VG AutoSpec using the technique described

by Tittlemier *et al.* (254). The exact mass of the molecular ion and fragments of the unknown compound were compared relative to the most probable theoretical compositions (Table 4.1). The mass differences between the theoretical masses and those of the unknown compound agreed to within 3 ‰ or better, confirming that the molecular formula was $C_9H_3OCl_7$, and that the first three fragment ions in the EI spectrum resulted from a loss of CH_3, Cl, and CH_3CO.

The molecular formula is indicative of a compound with five units of unsaturation. In conjunction with the low molecular weight, this suggests a monocyclic aromatic compound. A benzene ring accounts for four units of unsaturation, leaving the fifth unit of unsaturation to reside in a side chain double bond. The molecule has seven chlorine atoms and must contain a methoxy substituent. Thus, the most likely structure is a MeO-HpCS isomer. The *para* position is the most likely position on the phenyl ring for a hydroxyl group according to the mass spectrometry data. This position is also the most common location for the OH-group in OH-PCB compounds retained in plasma (5, 151, 231).

4.4. Synthesis of 4-hydroxy-heptachlorostyrene and Confirmation of Unknown Compound Identity

Based on the mass spectral evidence, it was probable that the unknown compound was 4-OH-HpCS, which has not been reported previously in the literature. Therefore, synthesis of 4-OH-HpCS was carried out by Wellington Laboratories (Guelph, ON, Canada by Alan McAlees).

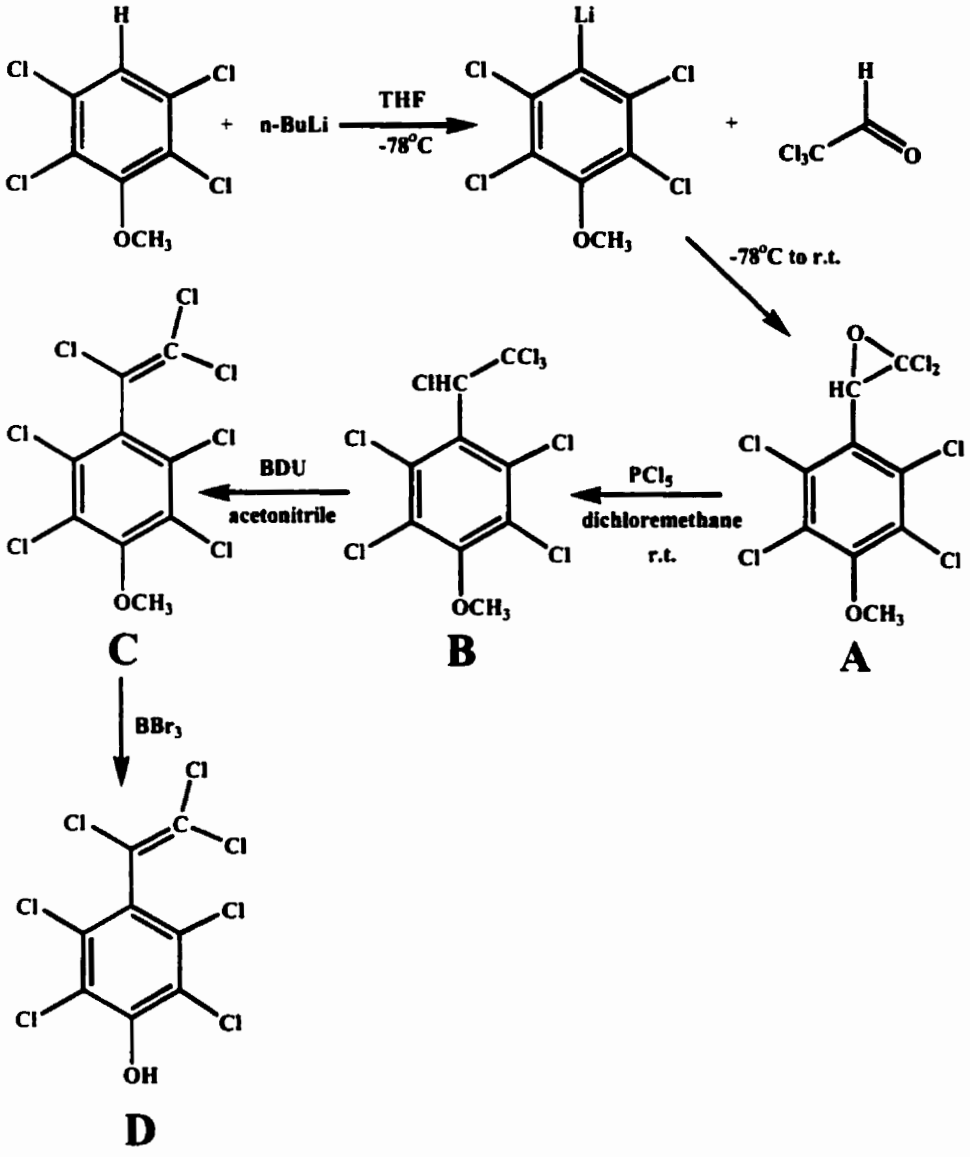


Figure 4.4 - Reaction scheme for the chemical synthesis of 4-OH-HpCS (r.t. - room temperature).

The reaction scheme for the preparation of 4-OH-HpCS is given in Figure 4.4. First 2,3,5,6-tetrachloroanisole was reacted with n-butyl-lithium in tetrahydrofuran at -78°C to generate the 4-methoxyheptachlorostyrene oxide. This was reacted immediately with chloral and allowed to warm to room temperature to generate the 4-

methoxyhepatchlorostyrene oxide (A). The yield for the reaction was approximately 31% after preparative TLC (silica gel, 25% dichloromethane/hexanes) which was applied to remove the remaining tetrachloroanisole and a pentachloroanisole impurity. The epoxide (A) was treated with excess phosphorus pentachloride (1.5-2.0 equivalents) in dichloromethane and allowed to react overnight at room temperature. The products of the reaction included 4-(1,2,2,2-tetrachloroethyl)-2,3,5,6-tetrachloroanisole (B) and MeO-HxCS. This mixture was heated for 4 hours on a steam bath with one equivalent of 1,5-diaza[5.4.0]-undecane (DBU) in acetonitrile to give 4-MeO-HpCS (C) and two other impurities. The MeO-HxCS was no longer detected. Compound C was isolated by thin layer chromatography and demethylated by refluxing with excess boron tribromide (6-7 equivalents, 1M in dichloromethane) in 1,2-dichloroethane for 20 hours. Compound D, 4-OH-HpCS, was separated from unreacted compound C using preparative TLC. The final purity of 4-OH-HpCS was greater than 99%.

Low-resolution EI and ECNI spectra for the 4-OH-HpCS were identical to those determined in the polar bear plasma samples (Figure 4.2). Furthermore, the exact mass assignments agreed with the unknown compound within 3‰ amu (Table 4.1). The retention time of the standard and the unknown compound were compared relative to the retention time of a major OH-PCB in polar bear plasma (4-OH-CB187) on three different GC columns of varying polarity: DB-5 ([5%-phenyl]-methylpolysiloxane from J&W Scientific Inc., Folsom, CA, USA, 30 m x 0.25 mm i.d., 0.25 μm film thickness), DB-1701 ([14%-cyanopropylphenyl]-methylpolysiloxane from J&W Scientific Inc., Folsom, CA, USA, 30 m x 0.25 mm

i.d., 0.25 μm film thickness), and RTX-2330 ([90%-biscyanopropyl-10%cyanopropylphenyl]-polysiloxane from Restek Corporation, Bellefonte, PA, USA, 30 m x 0.25 mm i.d., 0.20 μm film thickness). The relative retention time of the standard and compound present in the samples matched within experimental error on all three columns (Table 4.2).

Table 4.2 - Retention times of 4-OH-HpCS on three different GC columns relative to the retention time of 4-OH-CB187, one of the major OH-PCBs in polar bear plasma (see Figure 4.1).

	Relative Retention Time		
GC Column	Standard	Sample	difference
DB-5	0.7239	0.7245	-0.0006
DB-1701	0.6075	0.6079	-0.0004
RTX-2330	0.5914	0.5923	-0.0009

The unknown phenolic compound in polar bear plasma was therefore confirmed to be 4-OH-HpCS.

4.5. Transthyretin (TTR) Determination in Polar Bear plasma and Binding Affinity of 4-OH-HpCS to Human TTR.

TTR is a thyroid transport protein that is highly conserved among most mammals, birds and some reptiles (116). In order to confirm that polar bears possess TTR, polar bear plasma proteins (n=4) were separated by PAGE, as described previously by Brouwer and van den Berg (102). TTR confirmation and binding affinity experiments were completed elsewhere (Wageningen Agricultural University,

The Netherlands by Ilonka A.T.M. Meerts). Determination of ^{125}I-T4-competitive binding to specific proteins was performed as described by Lans *et al.* (58) and Darnerud *et al.* (172). In short, PAGE was performed with a 10% native separating gel with either pure polar bear plasma or plasma incubated with 100,000 counts/min of ^{125}I- T4. Purified TTR and bovine serum albumin were run as standards. After electrophoresis, part of the gel containing reference proteins were visualized by staining as described by Lans *et al.* (58). Parts of the gel containing plasma incubated with ^{125}I- T4 were sliced into 1 mm pieces and ^{125}I- T4 was measured by placing the slices in tubes and counting the radioactivity using a gamma counter. Plotting ^{125}I-T4-radioactivity against migration distance on the gel made the PAGE gel profile.

Three peaks showing TTR-bound radioactivity were identified in polar bear plasma after PAGE (Figure 4.5). Identification of the peaks was based on co-migration of the reference proteins and by comparing R*f*-values (i.e. the position of a protein on the gel (in mm) divided by the position of the front of the gel (in mm)) of the peaks containing radioactivity with R*f*-values of reference samples. R*f*-values of the two peaks containing radioactivity (R*f* = 0.45-0.49 and 0.62-0.63) are in accordance with Rf-values of the reference proteins, bovine serum albumin (R*f* = 0.41-0.42) and human TTR (0.59 -0.61), respectively. Thus, polar bears likely possess TTR. The last peak in Figure 4.5 represents free T4. As with other mammals, albumin is also present in polar bear plasma and the binding of T4 with albumin is higher than TTR.

OH-PCBs have been shown to bind with high affinity to TTR and not other thyroid hormone transport proteins, such as thyroxine binding globulin (142, 258).

Most OH-PCB congeners detected in plasma have a hydroxyl group in the *para*-position relative to the phenyl-phenyl bond and have adjacent chlorines in both meta-positions (5, 151, 231). Since 4-OH-HpCS is structurally similar to other chlorinated phenolic compounds that bind with high affinity to TTR and TTR was found in polar bear plasma, it was of interest to determine the relative binding affinity of 4-OH-HpCS and T4 to TTR.

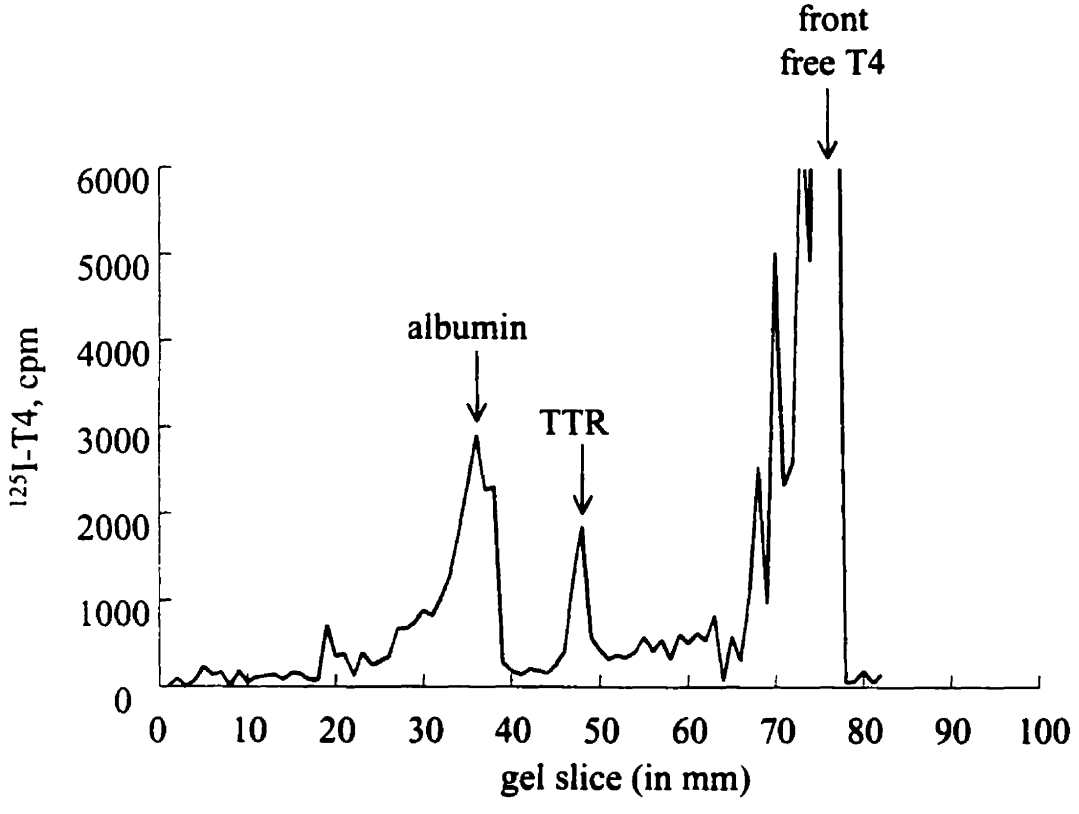


Figure 4.5 - Separation by polyacrylamide gel electrophoresis (PAGE) and radioactive detection of proteins in polar bear plasma after incubation with ^{125}I-thyroxine (T4).

The assay for determining competitive binding of 4-OH-HpCS and ^{125}I-labeled T4 to human TTR was performed as described elsewhere (58). Briefly, the assay

mixture contained 30 nM human TTR, a mixture of ^{125}I- labeled and unlabeled T4 (70,000 cpm, 55 nM), and the competitor (T4 or 4-OH-HpCS in increasing concentrations ranging from 10^{-9} to 10^{-4} M) dissolved in 0.1 M Tris-HCl-buffer (pH 8.0, containing 0.1 M NaCl and 0.1 mM EDTA). The incubation mixtures were allowed to reach equilibrium and the protein bound and free ^{125}I- T4 were separated on Biogel P-6DG columns and eluted. The radioactivity in the eluate was determined by gamma counting and compared to control incubations. Concentration-dependent, competitive binding curves of T4 and 4-OH-HpCS relative to ^{125}I- T4 (% of control) for TTR protein are shown in Figure 4.6.

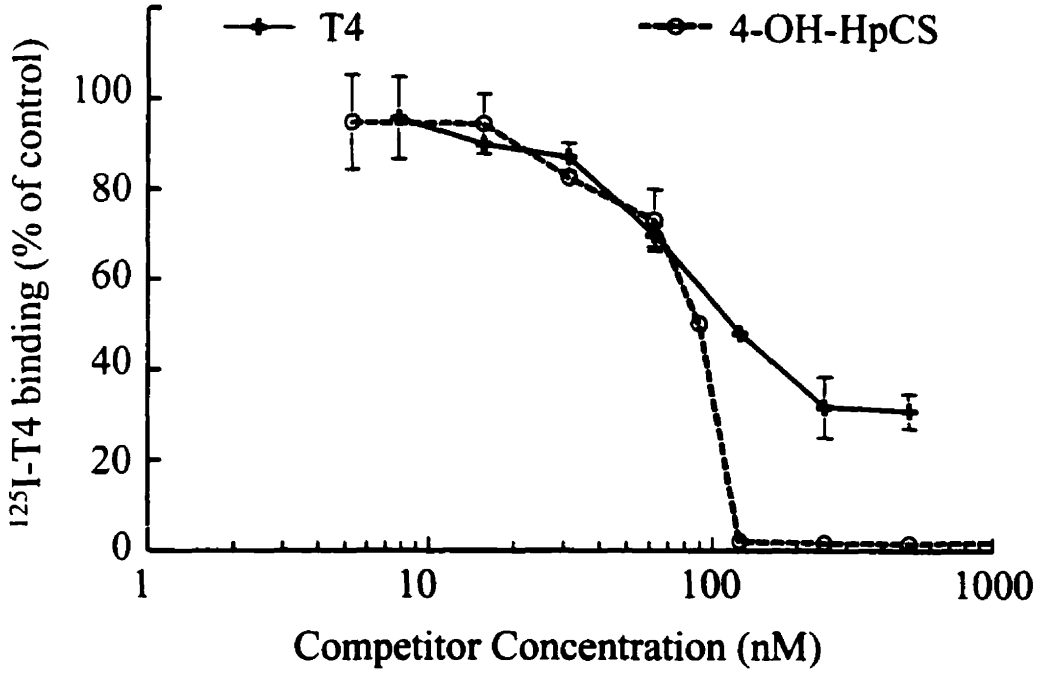


Figure 4.6 - The concentration-dependent, competitive binding of 4-OH-HpCS and T4 relative to ^{125}I-T4 for human transthyretin (TTR). Assays were performed in duplicate.

IC_{50} values (concentration of competitor at half maximal specific binding) and relative binding affinities (RBA) were calculated as described by Meerts *et al.* (145).

RBA was calculated by dividing the IC_{50} of T4 by the IC_{50} of 4-OH-HpCS. The competitive binding assay was done in duplicate and within the assay each concentration was tested in triplicate.

The RBA and IC_{50} values of 4-OH-HpCS were 3.96 x 10^7 M^{-1} and 71.74 nM, respectively. The relative potency of 4-OH-HpCS versus T4 for TTR binding was 1.1. This is within the range of potencies of other halogenated phenolic compounds reviewed by Brouwer *et al.* (136) and Brucker-Davis (137) and indicates that circulating levels of 4-OH-HpCS in blood have the potential to disrupt thyroid hormone transport and possibly retinol transport *in vivo*. The displacement of the natural ligand (T4) from TTR by halogenated phenolic compounds is hypothesized to result in an increased clearance of plasma T4 *in vivo*. This has been observed in animals exposed to CHCs such as PCBs (172) or hydroxylated PCBs (246). In another example, Sinjari *et al.* (171) demonstrated that administration of 4′-OH-3,3′,4,5′-tetrachlorobiphenyl and 4-OH-2,3,3′,4′,5-pentachlorobiphenyl to pregnant mice reduced the total T4 levels in both maternal and fetal plasma. Recent results indicate that pregnant rats dosed (oral) with 4-OH-CB109 had decreased T4 levels (173). However, the fetuses (gestational day 20) showed the greatest effects with FT4 and TT4 levels decreased 98 and 41 % as compared to controls (173). Thus, phenolic metabolites of CHCs may be very important in the thyroidogenic properties observed with CHC exposure, especially those exposed *in utero*.

4.6. Concentrations of 4-OH-HpCS Compared to Other Chlorinated Phenolic Compounds, OCS and PCBs

Thirty polar bear plasma samples were analyzed for chlorinated phenolic compounds, PCBs and OCS. The polar bears included both males and females (15 each) and ranged from 1 to 27 years of age. The plasma samples (3.04 - 4.21 grams) were extracted and analyzed as described in Chapter 3.

OH-PCBs and PCP were quantified by gas chromatography ECNI-MS, employing authentic standards when available or by using group response factors for unidentified compounds as described in Chapter 3. PCBs were quantitated by gas chromatography EI-MS using a characterized secondary polar bear quantitation standard (PBQ) (235) as described in Chapter 3. Concentrations of 4-OH-HpCS were determined separately by GC-ECD (conditions as described above) using a calibration curve of the authentic standard. The total concentration of chlorinated phenolic compounds was calculated from the sum of hydroxy-PCBs (ΣOH-PCBs, 37 congeners), PCP and 4-OH-HpCS. ΣPCBs comprised the sum of 23 congeners (235). The OH-PCB data is presented only to illustrate the significance of the 4-OH-HpCS with respect to the other major chlorinated phenolic compounds. Further interpretation of the OH-PCBs found in polar bear plasma will be discussed in Chapter 5.

Concentrations of 4-OH-HpCS, ΣOH-PCBs, PCP, OCS, CB153 and ΣPCBs in polar bear plasma are listed in Table 4.3. Plasma concentrations of 4-OH-HpCS were on average 39 times higher than OCS, although the ratio of 4-OH-HpCS to OCS

ranged from 5.19 to 216. The mean ratio of 4-OH-HpCS to CB153 was 0.7 (± 0.6 SD), indicating that 4-OH-HpCS is one of the main contaminants in polar bear plasma.

Concentrations of 4-OH-HpCS constituted on average 12.2% of the total concentration of chlorinated phenolic compounds in polar bear plasma, the remainder being almost entirely OH-PCBs (Table 4.3, Figure 4.1). 4-OH-HpCS was similar in concentration to the major OH-PCB congeners. The concentration of ΣPhenolic compounds was approximately twice that of ΣPCBs and is likely caused by the high binding affinity of the phenolic compounds to plasma proteins. PCP was a small percentage of total chlorinated phenolic compounds, unlike in humans, where it is frequently the most important contributor (151).

Table 4.3 - Plasma concentrations (ng/g wet weight) and selected mean ratios of concentrations of chlorinated phenolic compounds, including 4-OH-HpCS and related CHCs in polar bear plasma (n=30) from the Resolute Bay area, Nunavut Territory, Canada and ringed seal plasma (n=5) from Kuujjuaq, Quebec, Canada. (min. = minimum, max. = maximum, SD = standard deviation)

	Polar Bear Plasma				Ringed Seal Plasma	
	mean	min.	max.	SD	mean	SD
4-OH-HpCS	9.11	2.89	22.9	3.85	0.062	0.023
PCP	0.210	0.093	0.531	0.099	0.237	0.136
Σ OH-PCBs	92.6	26.4	576	117	0.081	0.042
Σ Phenolics	103	33.2	600	120	0.379	0.181
OCS	0.348	0.106	0.940	0.188	0.266	0.086
CB 153	20.2	4.81	82.0	16.3	5.16	3.71
Σ PCBs	46.9	16.1	161	32.3	27.1	16.5
4-OH-HpCS/Σ Phenolics	0.122	0.038	0.248	0.053	0.176	0.077
4-OH-HpCS/OCS	39.2	5.19	216	41.2	0.260	0.124
4-OH-HpCS/CB153	0.712	0.035	2.43	0.580	0.016	0.009
OCS/CB153	0.024	0.005	0.078	0.018	0.072	0.042

4.7. Evidence for Bioaccumulation and Metabolism of OCS as a Source of 4-OH-HpCS in the Polar Bear Food Chain

Long-range transport from source regions and bioaccumulation of 4-OH-HpCS in the polar bear food chain is not likely. First, chlorophenols generally have high water solubility and low volatility. Therefore, they would be prone to remain in areas close to sources (259). Halogenated phenols have not been reported in any Arctic biota (63). Second, phenolic compounds are readily conjugated and excreted in many higher organisms and are not expected to biomagnify in mammal food chains (260). Thus, the most probable sources of 4-OH-HpCS are through metabolism of OCS or a heptachlorostyrene (HpCS) congener.

Isomers of HpCS with unknown chlorine substitution pattern have been reported in the literature, at levels 10 fold less than OCS in the Elbe river in Germany and the Great Lakes where OCS contamination is relatively high (261, 262). HpCS congeners have never been observed in Arctic biota and are unlikely sources of 4-OH-HpCS. There can be little doubt that 4-OH-HpCS in polar bear plasma results from CYP450-mediated metabolism of OCS. Very little is known regarding the dechlorination mechanism of fully chlorinated aromatic compounds. HCB has been shown to form PCP when rats were exposed *in vivo* (70) and was latter shown to involve the CYP450 3A isozymes during *in vitro* studies (263). These may be the same class of enzymes involved in the dechlorination of OCS to heptachlorostyrene as seen when rats were dosed *in vivo* (249).

Table 4.4 - Mean concentrations of CB153 and octachlorostyrene (ng/g lipid weight) and mean ratios in polar bear liver and adipose samples (mean ± SD).

Tissue	Sample Size	OCS	CB-153	Ratio OCS:CB-153
Polar Bear Adipose	8	14 ± 12	2670 ● 640	0.005 ± 0.005
Polar Bear Liver	8	156 ± 115	6840 ± 3120	0.022 ± 0.008

OCS was identified in polar bears as part of another study but not reported (230, 251). In order to compare relative accumulation of OCS and CB-153 in plasma with that in adipose tissue and liver, concentrations of OCS and CB-153 from these previous studies were re-examined. A description of analytical methods and concentrations of PCBs, DDE and their methylsulfone metabolites in polar bear adipose tissue and liver are given by Letcher *et al.* (230, 252). OCS concentrations, which were simultaneously quantitated by GC/EI-MS in that study, are reported in Table 4.4. OCS was found to be a relatively minor contaminant in liver and adipose tissue, as was the case for plasma. Tissue-specific accumulation of OCS occurred in liver since the ratio of OCS to CB-153 in polar bear liver was four times that of fat ($p < 0.001$). The ratio of OCS to CB-153 in liver was nearly identical to that in plasma of bears from the same area (Table 4.3). Given the high variance in the ratios, this finding is probably a coincidence. Nevertheless, it suggests that plasma concentrations of OCS relative to CB153 in polar bears are reflective of other tissues.

Five ringed seal plasma samples from around Kuujjuaq in northern Quebec were analyzed as described above and results are shown in Table 4.3. Sample sizes ranged from 3 to 7 grams and ages were not known. Mean recoveries of the ^{13}C-

labeled OH-PCBs, PCBs, and PCP were 110% ± 8% CV, 74 ± 4% CV and 71 ± 10% CV, respectively. The ringed seals were sampled from a region that is a considerable distance from where the polar bear samples were taken. However, differences in patterns and concentrations of CHCs (including PCBs) in seals and bears between these areas has been determined to be relatively small (235, 264, 265). Therefore, geographical differences in contamination are not expected to influence the comparison between species.

Concentrations of 4-OH-HpCS were 147 times lower in seals than in polar bears even though OCS plasma concentrations were only slightly lower in seals. In seals, 4-OH-HpCS constituted 17.6 % of the total concentration of the chlorinated phenolic compounds, which was similar to the polar bear samples (12.2%). The ratio of 4-OH-HpCS to OCS was 150 times lower in seal than polar bear. This demonstrates that seals are capable of metabolizing OCS to 4-OH-HpCS but at a much slower rate than polar bears. The mean concentration of total phenolic compounds was 272 times lower in seals than bears. These findings support previous data that showed ringed seals have a lower capability to metabolize CHCs than polar bear and other terrestrial mammals (230).

Despite a large difference in ratios of 4-OH-HpCS to OCS, the mean ratio of OCS to CB153 was only three times higher for seal than polar bear. This suggests that while formation/retention of 4-OH-HpCS is much more rapid in bears than in seals, it is still slow compared to net bioaccumulation of OCS in the bear. The situation is similar to that for PCBs. Most of the major OH-PCBs in human plasma are believed to be formed by metabolism of highly recalcitrant PCBs, such as CB118, CB138 and

CB153, but this rate of metabolism is insignificant compared to rate of accumulation and loss by other mechanisms, such as partitioning into fecal matter (231).

4.8. Discussion

OCS is an industrial by-product. The major source of OCS is thought to involve electrolysis of salt solutions using a carbon electrode, especially in the production of sodium hydroxide and chlorine from sodium chloride (266). Other possible sources include emissions from the purification of aluminum with gaseous chlorine in graphite vessels (267) and industrial processes involving the electrolysis of magnesium chloride (268). OCS is generally a low-level environmental contaminant that is usually considered to be of little significance compared to major persistent CHCs, especially PCBs. Although there are few publications that report OCS concentrations, it is a global contaminant found in arctic fish (268) and Antarctic seabirds (269). OCS has been found to accumulate to high concentrations in fish from the Frierfjord in Norway (268) and the Great Lakes (262), close to local sources.

OCS is not a major CHC in Arctic marine food webs (63). Therefore, the relatively high concentration of 4-OH-HpCS in polar bear plasma compared to other phenolic compounds (mainly OH-PCBs), as well as other major contaminants (such as CB153), was unexpected. The presence of 4-OH-HpCS in polar bear and ringed seal plasma is most reasonably explained by metabolism of OCS. At least in some species at higher trophic levels, the findings from the present study suggest that the significance of OCS as an environmental contaminant may have been underestimated.

4-OH-HpCS is a phenolic compound retained in plasma and thus bioaccumulation from seal to bear is not an influencing factor since seal fat and not blood is the important component in the polar bear diet. Concentrations of 4-OH-HpCS and relative concentrations to CB153 and OCS were much lower in ringed seal relative to polar bear plasma. This is probably because of lower capacity of seals to metabolize CHCs (230). OCS/CB153 ratios were similar in plasma of the two species, suggesting that rate of formation of 4-OH-HpCS in the polar bear is not fast compared to the rate of accumulation of OCS from the diet. The apparent anomaly of relatively high levels of metabolites of slowly metabolized CHCs in plasma presumably occurs because of specific and high binding affinity of the metabolites to plasma proteins (231). If this binding is strong enough, it will compete with conjugation and excretion mechanisms in liver and kidney. Thus, the metabolites are effectively transferred into the plasma compartment and protected from excretion.

Transthyretin (TTR) is assumed to be the main plasma protein responsible for specific binding of phenolic compounds found in plasma because most of the compounds, including 4-OH-HpCS, have a similar structure to the natural ligand, the thyroid hormone, T4 (58). We demonstrated that TTR was present in polar bear plasma, therefore, binding to this protein is a plausible explanation for the relatively high levels of 4-OH-HpCS in these species.

The binding affinity of 4-OH-HpCS to human TTR was approximately the same as T4. However, several OH-PCBs, PCP and even PCBs such as CB153 (144) have been shown to bind with even greater relative binding affinity to T4 (72, 142). Little is known about differences in binding affinity of T4 or metabolites of CHCs to

TTR among species except for humans and laboratory animals. Nor is anything known about the possibility that other proteins, such as albumin, may participate in plasma binding of these compounds in some species. Therefore, it is unclear how important a role TTR plays in maintaining high concentrations of halogenated phenolic compounds or in the transport of T4, in the plasma of mammals. These are important factors in understanding possible disruption of thyroid hormone and retinol homeostasis. We discuss the effects of contaminants and their metabolites on circulating thyroid hormone and retinol levels in the polar bear (Chapter 5) to help resolve some of these questions.

4.9. Acknowledgements

Funding for this research was supplied in part by the Northern Contaminants Program, the Canadian Chlorine Coordinating Committee (C4) and the Canadian Chemical Producers Association. I would like to thank Mary Simon (Canadian Wildlife Service, Environment Canada) for her help and technical assistance in the use of the high-resolution mass spectrometer. The polar bear adipose and liver data was supplied by Dr. Robert Letcher (GLIER, University of Windsor, Windsor, ON, Canada). Synthesis and purification of 4-OH-HpCS was completed by Alan McAlees and Brock Chittim from Wellington Laboratories (Guelph, ON, Canada). Dr. Abraham Brouwer and Ilonka Meerts supplied the TTR determination and binding affinity measurements.

Chapter 5. CHCs as possible disruptors of thyroid hormone and retinol homeostasis in polar bear plasma: Identification and role of OH-PCBs

5.1. Background

Polar bears reside atop the Arctic food chain. Their diet predominantly consists of ringed seal blubber (270) and through dietary accumulation, they are exposed to many exogenous compounds (64). Exposure to high concentrations of CHC contaminants has resulted in elevated body burdens of chlordanes and PCBs in the polar bear. The increased body burden of these CHCs appears to correlate with enhanced metabolic capability (230) as a result of enzyme induction (252). High levels of CHCs and suspected metabolic capability suggest that PCB metabolites may be important contaminants in polar bears. Methylsulfone-PCBs have been shown to accumulate in polar bears (230), but nothing is known about hydroxy metabolites of PCBs (OH-PCBs).

Some OH-PCBs affect both thyroid hormone and retinol homeostasis, which are both commonly perturbed endpoints associated with PCB exposure (92, 93, 156). The toxicological properties of OH-PCBs make these a potentially important group of contaminants and there is a clear need to assess their concentrations and effects in the environment. OH-PCBs have been analyzed in a limited number of species and quantitated in even fewer. OH-PCBs have only been quantitated in human (151, 192) and White-tailed sea eagle plasma (205).

Since polar bears are exposed to large amounts of PCBs and have proven high activity to metabolize many congeners (230), they were a good species to test for associations between OH-PCBs and retinol and thyroid hormone concentrations, which are commonly used biomarkers of CHC exposure and possible effects.

There have only been two published studies on thyroid hormone concentrations in polar bears. Leatherland and Ronald (271) analyzed captive polar bears for seasonal differences and feral polar bears for gender and age differences in thyroid hormone concentrations. They determined that gender, age and season could affect thyroid hormone concentrations. Thus, these factors must be considered during statistical analysis. Skaare *et al.* measured thyroid hormones, retinol and CHCs in Svalbard polar bears (272). They found that none of the CHCs were correlated with plasma T3 or T4 concentrations but the ratio of TT4:FT4 and retinol concentration decreased linearly with increasing concentrations of HCB and PCBs.

This initial study compares the concentrations of OH-PCBs and other common contaminants as well as thyroid hormone measures and retinol concentrations between polar bear populations from two regions. Polar bears from Svalbard, Norway and Resolute Bay, Nunavut Territory, Canada were chosen to represent high and low exposure to PCBs. It has been shown that Svalbard bears have higher PCBs concentrations than Resolute bears (235, 273, 274). Letcher *et al.* (251) noted variation in PCB patterns in adipose tissue from polar bears sampled at different locations. An increasing CB99 to CB180 ratio from west to east demonstrated that higher chlorinated congeners were more abundant in polar bears in Greenland as compared to Alaska. This trend likely continues in polar bears from Svalbard since it

was shown that the more recalcitrant hexa- and pentachloro congeners made up higher proportions of the PCB pattern in European arctic ringed seals as compared to Canadian arctic ringed seals (265). Thus, investigation of OH-PCB metabolites from Svalbard and Resolute Bay polar bears might show pattern differences as well as possible effects on thyroid hormone and retinol homeostasis.

5.2. Materials and Methods

Seventy-one polar bear plasma samples were chosen for analysis from a larger set of samples and analyzed for OH-PCBs and other CHCs at random. Samples included males and females ranging in age from less than 1 year old to 27 years of age. Vestigial premolar tooth extraction allowed measurement of age as described by Calvert and Ramsay (275).

Thirty-three of the polar bears were captured between April and May 1997 by the late Dr. Malcolm Ramsay (University of Saskatchewan, Saskatoon, SA) around the Resolute Bay area, Lancaster Sound, Nunavut Territory, Canada (Figure 5.1). The other thirty-eight polar bears were captured between April and May 1998 by Dr. Andrew E. Derocher (Norwegian Polar Institute, Tromsø, Norway) and Dr. Øystein Wiig (Zoological Museum, University of Oslo, Norway) near Hopen and Edgeøya Islands in southeast Svalbard, Norway (Figure 5.1). For the Canadian samples, whole blood was drawn into heparinized vacutainers (50 ml) and stored on ice and out of light until processed. Blood samples were centrifuged and plasma drawn off, frozen at

– 40°C and stored until further analysis. For Norwegian samples, blood samples were stored in cooler (not on ice) and were processed in a similar manner.

Polar bear plasma samples were extracted and quantitated using the methods and techniques described in Chapter 2 and 3. Over 75 individual compounds were quantitated in the polar bear plasma samples. The main CHCs determined included 33 halogenated phenolic compounds (mostly OH-PCBs), 24 PCB congeners, 10 chlordane compounds, 3 chlorinated benzenes, DDT and metabolites and α/β HCH.

Plasma retinol concentrations were determined as described by Honour *et al.* (276). This method does not include the analysis of retinyl esters as described in a recent publication that included total polar bear plasma retinol concentrations (272). Thyroid hormone measurements were completed by Mitra Brown and Scott Brown (National Water Research Institute) using methods as described elsewhere (277). Briefly, total T3 and total T4 were determined by radioimmunoassay. Free T3 or T4 indices were determined by measuring the relative capacity of each plasma sample to bind and elute ^{125}I-T3 or ^{125}I-T4 from a miniature Sephadex column. In fish, the FT4 index has been shown to be highly correlated with FT4 concentrations, which are much more difficult to determine directly (270). The FT4 index is therefore a surrogate for FT4 concentrations. Columns were equilibrated at room temperature and washed with a sodium hydroxide solution (0.1 N) and drained. Labeled thyroid hormone (^{125}I) was added to each column in a sodium hydroxide solution (0.1 ml, 0.1 N) followed by diluted plasma (1 ml). The dilution factor varied with experiments but was approximately 15 fold and 80 fold for free T3 and free T4 indices, respectively. The columns were then washed with phosphate buffer (3 ml) to remove plasma

proteins and labeled hormones counted. Free thyroid hormone indices were calculated using the following formula (cpm – counts per minute):

$$\text{FT Index} = \frac{\text{(total hormonal cpm added to column – eluted cpm x 100)}}{\text{(total hormonal cpm added to column x dilution factor)}}$$

All statistical analysis was completed with STATISTICA for Windows - version 5.1 from StatSoft, Inc. (1997) (Tulsa, OK). Chemical residue data was not normally distributed, therefore, data was log transformed prior to statistical analysis. Retinol, T3, and T4 concentrations were also log transformed free T3 and T4 indices were not log transformed since they represent a ratio of binding capability and were normally distributed among the bears. Correlations were computed using Pearson Product moment calculations. Regional and gender differences were determined by the student's t-test ($p<0.05$).

Principal component analysis was completed on log transformed data using Varimax normalized rotation. This rotation is aimed at maximizing the variances of the squared normalized factor loadings across variables for each factor and is equivalent to maximizing the variances in the columns of the matrix of the squared normalized factor loadings.

5.3. Results and Discussion

5.3.1. Thyroid hormones and retinol

Polar bears were categorized into eight groups based on population (Svalbard and Resolute), gender and age. The thyroid hormone and retinol results are given in Table 5.1. Due to lactational exposure to CHCs, polar bears between 0 and 2 years old (cubs) have been shown to have higher concentrations of CHCs than adult bears. Between 3 and 4 years old (juveniles), their levels start to decrease, approaching those of adults (278). Thus, in order to compare biochemical and organochlorine data, cubs and juveniles were excluded from further statistical analysis because of potential effect of age on thyroid hormone and retinol homeostasis. Leatherland and Ronald (271) showed that juvenile bears had more variation in their thyroid hormone concentrations but due to the limited number of samples, they were unable to determine if concentrations were significantly different. Data for cubs and juveniles are shown only for comparison to adult levels. Norstrom and Muir (235) suggested that concentrations of PCBs and chlordanes decrease by a factor of 2 for both genders until five years of age, thus supporting the exclusion of sub-adults from this data set.

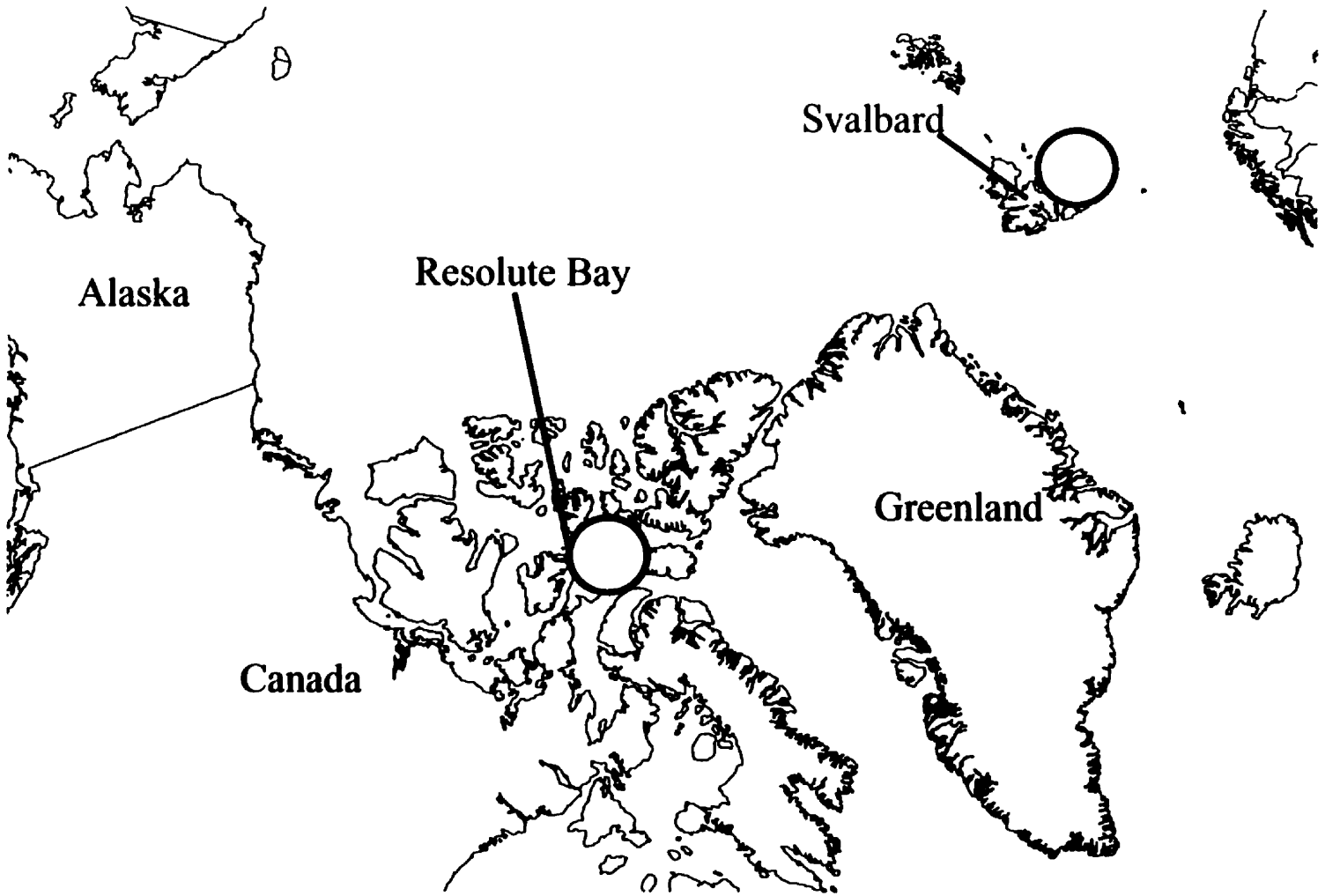


Figure 5.1 - Locations of polar bears sampled as part of study to examine the effects of OH-PCBs and other CHCs on thyroid hormone and retinol homeostasis – near Resolute Bay, Nunavut Territory, Canada and near Hopen and Edgeøya Islands in southeast Svalbard, Norway.

Table 5.1 - The biochemical measures determined for each of the polar bear plasma samples. Polar bears are separated by region, age and gender for comparison.

	Resolute Cubs (N=3)		Resolute Juveniles (N=5)		Resolute Male Adults (N=12)		Resolute Female Adults (N=13)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
age (years)	-	-	-	-	10	4	14	7
T3 (nmol/l)	0.21	0.21	0.08	-	0.19	0.19	0.15	0.08
Free T3 Index	4.57	1.74	2.91	0.95	5.19	1.34	2.81	1.59
T4 (nmol/l)	8.00	2.75	6.25	0.41	4.93	4.05	6.62	2.06
Free T4 Index	1.08	0.09	1.17	0.03	1.13	0.04	1.15	0.04
Retinol (µmol/l)	1.16	0.33	1.17	0.50	0.70	0.20	1.08	0.46
	Svalbard Cubs (N=3)		Svalbard Juveniles (N=2)		Svalbard Male Adults (N=18)		Svalbard Female Adults (N=15)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
age (years)	-	-	-	-	14	6	14	4
T3 (nmol/l)	0.23	0.13	0.69	0.67	0.34	0.30	0.59	0.36
Free T3 Index	6.18	0.15	6.24	0.05	6.31	0.08	6.25	0.09
T4 (nmol/l)	1.15	1.70	0.39	0.25	2.33	1.87	3.88	4.82
Free T4 Index	0.79	0.11	0.75	0.14	0.93	0.18	0.84	0.18
Retinol (µmol/l)	1.22	0.26	0.88	0.43	0.79	0.34	0.96	0.29

For adult bears, all thyroid hormone measures were significantly different ($p<0.05$) between regions suggesting that thyroid hormone measurements may be associated with the different contaminant levels found in the two populations (Table 5.2). Resolute bears showed higher total T4, free T4 binding index while showing lower total T3 and free T3 index than Svalbard bears. Retinol concentrations were not significantly different between regions. None of the biological measures were

significantly related to age, even when separated into region and gender categories. This is in contrast to a recent study which found that total T4, free T4, total T3 and free T3 are associated with age in male polar bears (272). Gender differences in retinol and thyroid hormone measures for each of the populations are also shown in Table 5.2. Only the free T3 index was statistically different in males and females from both populations. Therefore, the free T3 index was sex corrected prior to statistical analysis. As found in a previous study on Svalbard bears (272), females had higher total T4 than males in both populations. Total T3 was only higher in females for the Svalbard population as found previously (272). There seems to be some discrepancy in the quantitation of total T4 concentrations. There are now three studies that examine thyroid hormones in polar bears. The range of concentrations of total T4 for adult male polar bears were 74 nmol/l (mean)(Churchill – October) (271), 8-25 nmol/l (Svalbard – March) (272), 1.7-7.64 nmol/l (Svalbard – March) and 5.40-14.6 nmol/l (Resolute Bay – March, Table 5.1). Seasonal variations have been shown by Leatherland and Ronald (271) but the fluctuations were quite small. Thus, T4 concentrations may not be comparable between studies but the measurements can still be used for comparisons with chemical residues for this study because consistent methodology was used throughout. Retinol and T3 concentrations for Svalbard bears in this study and that described by Skaare *et al.* (272) were similar, thus allowing comparison of concentrations to be made.

Table 5.2 - Population and gender differences for the biochemical measures in the adult polar bears shown below. Higher and lower concentrations are denoted by + and - signs. The p-value is given when statistically different.

Biochemical Measure	Adult Resolute Bears	Adult Svalbard Bears	p value	Svalbard Bears			Resolute Bears		
				males	females	p value	males	females	p value
T3 (nmol/l)	+	-	0.002	+	-	0.02	-	-	-
Free T3 Binding Capacity	-	+	< 0.001	+	-	0.04	+	-	< 0.001
T4 (nmol/l)	+	-	0.001	-	-	-	-	-	-
Free T4 Binding Capacity	+	-	< 0.001	-	-	-	-	-	-
Retinol (µmol/l)	-	-	-	-	-	-	-	+	0.008

5.3.2. Chlorinated hydrocarbons

The concentrations of chlorobenzenes in all bear groups are shown in Figure 5.2. Svalbard bears have statistically lower tetra- (TeClBz) and pentachlorobenzene (PnClBz) concentrations (both $p<0.005$) but higher hexachlorobenzene (HCB) concentrations ($p=0.007$) than Resolute bears. The higher HCB concentrations in Svalbard bears than Resolute bears are similar to results described for ringed seal samples by Muir et al. (227). Sub-adults had higher concentrations than adults and followed the same pattern as adults, with Svalbard bears having higher concentrations of HCB ($p=0.04$) and lower concentrations of tetrachlorobenzene and pentachlorobenzene (both $p=0.01$). It is not known why Resolute bears have higher concentrations of TeClBz or PnClBz compared to Svalbard bears. Penta- and hexachlorobenzene concentrations are important contaminants to determine, as they are possible precursors to pentachlorophenol (PCP), which is found in the phenolic compound fraction.

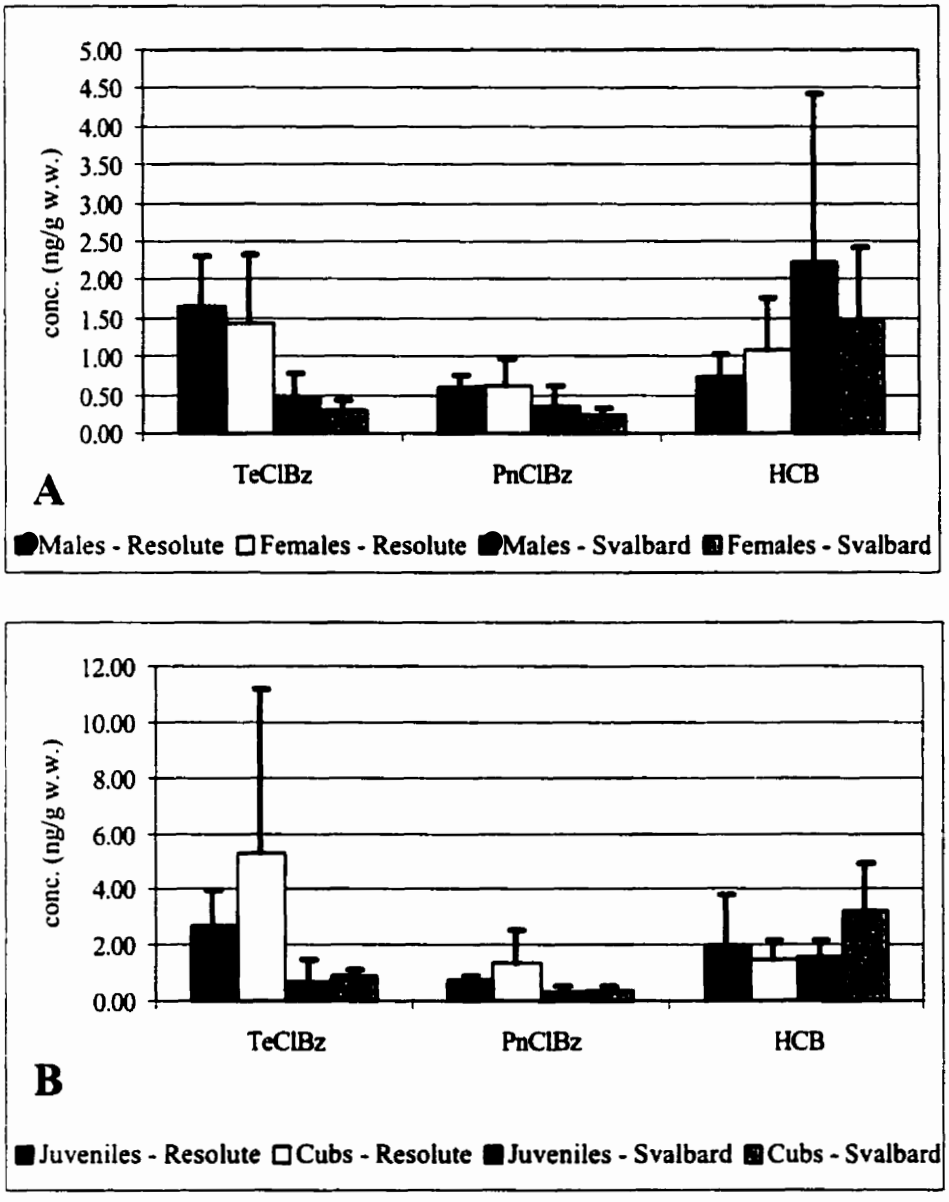


Figure 5.2 - Mean chlorobenzene concentrations (ng/g wet weight) determined in polar bear plasma samples. A - adults only, B - sub-adults (error bars = standard deviation).

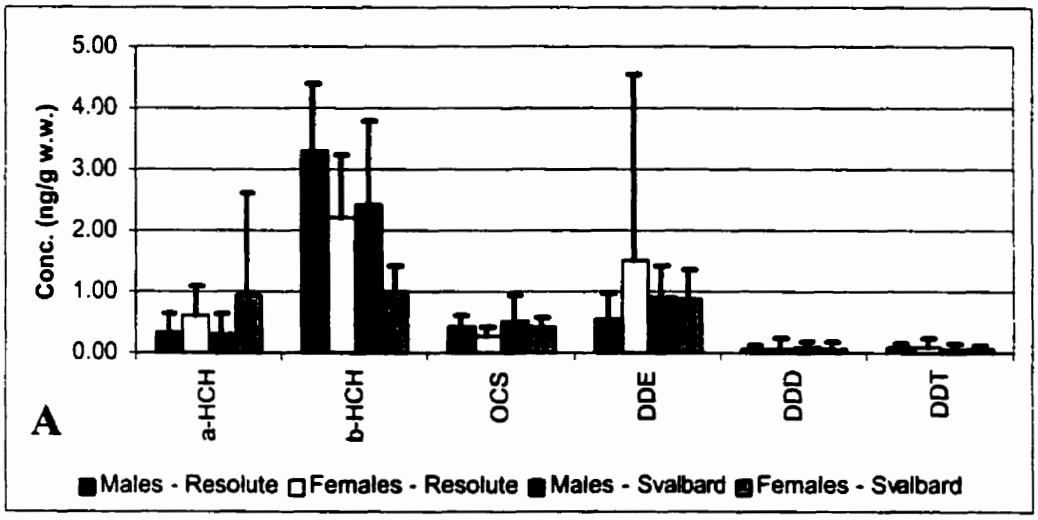
Concentrations of α-HCH concentrations (Figure 5.3) were similar between regions but were higher in females than males. β-HCH concentrations were 9 times

higher than α-HCH and male bears had higher β-HCH concentrations than females in both populations. This has been described previously and is thought to be a result of lactation and the high HCH levels associated with milk (278). Resolute bears were found to have statistically higher concentrations ($p = 0.006$) of ΣHCH congeners as described previously by Muir and Norstrom (279). The difference in HCH concentrations between regions was especially apparent for sub-adults (Figure 5.3) as Resolute bears had significantly higher ΣHCH than Svalbard bears.

DDT and its metabolites (DDE and DDD) were not significantly different between regions or gender (ΣDDT), as implied in Figure 5.3. This may be due to the large variation found for DDE in Resolute females. No differences in DDE levels between genders were found by Bernhoft *et al.* (278), which was attributed to low persistence of DDE in polar bears (235). This is further supported by the fact that body burdens of DDT and metabolites decrease during fasting (280, 281), which is an indication of metabolism and excretion.

OCS is often determined in biological samples as part of routine protocols but concentrations are rarely reported in the literature. With the discovery of a likely metabolite, 4-hydroxyheptachlorostyrene, in the phenolic compound fraction of polar bear plasma (Chapter 4) (147), more information is needed about this relatively unknown compound. Resolute females had the lowest concentration of OCS and females from both regions had lower concentrations than males (Figure 5.3). Little is known about sources of OCS to the Arctic environment, as it has not been extensively studied. There have been sources of OCS determined in Norwegian fjords that occur

due to processes involving the electrolysis of magnesium chloride (268), which may explain the slightly higher levels in Svalbard bears.



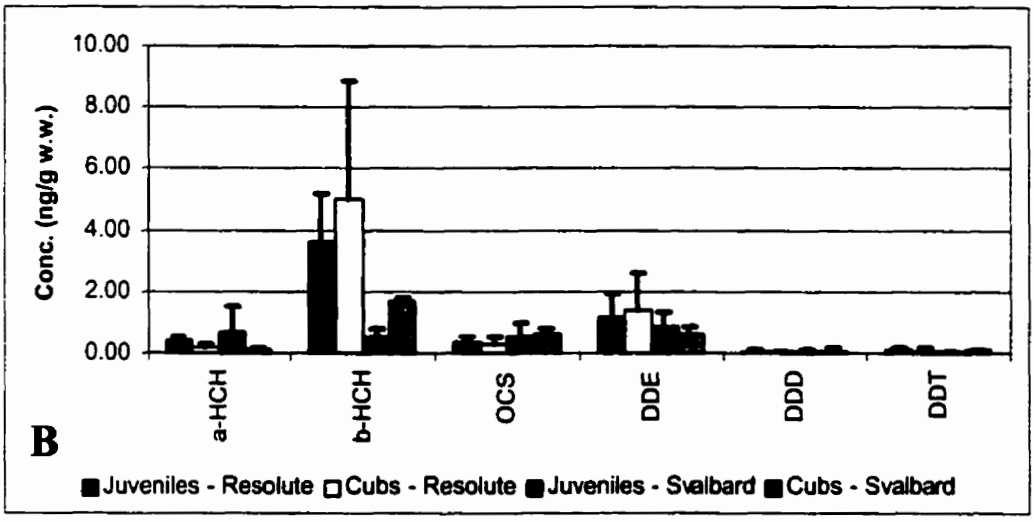


Figure 5.3 - Mean OCS, HCHs and DDT concentrations (ng/g wet weight) determined in polar bear plasma samples. A - adults only, B - sub-adults (error bars = standard deviation).

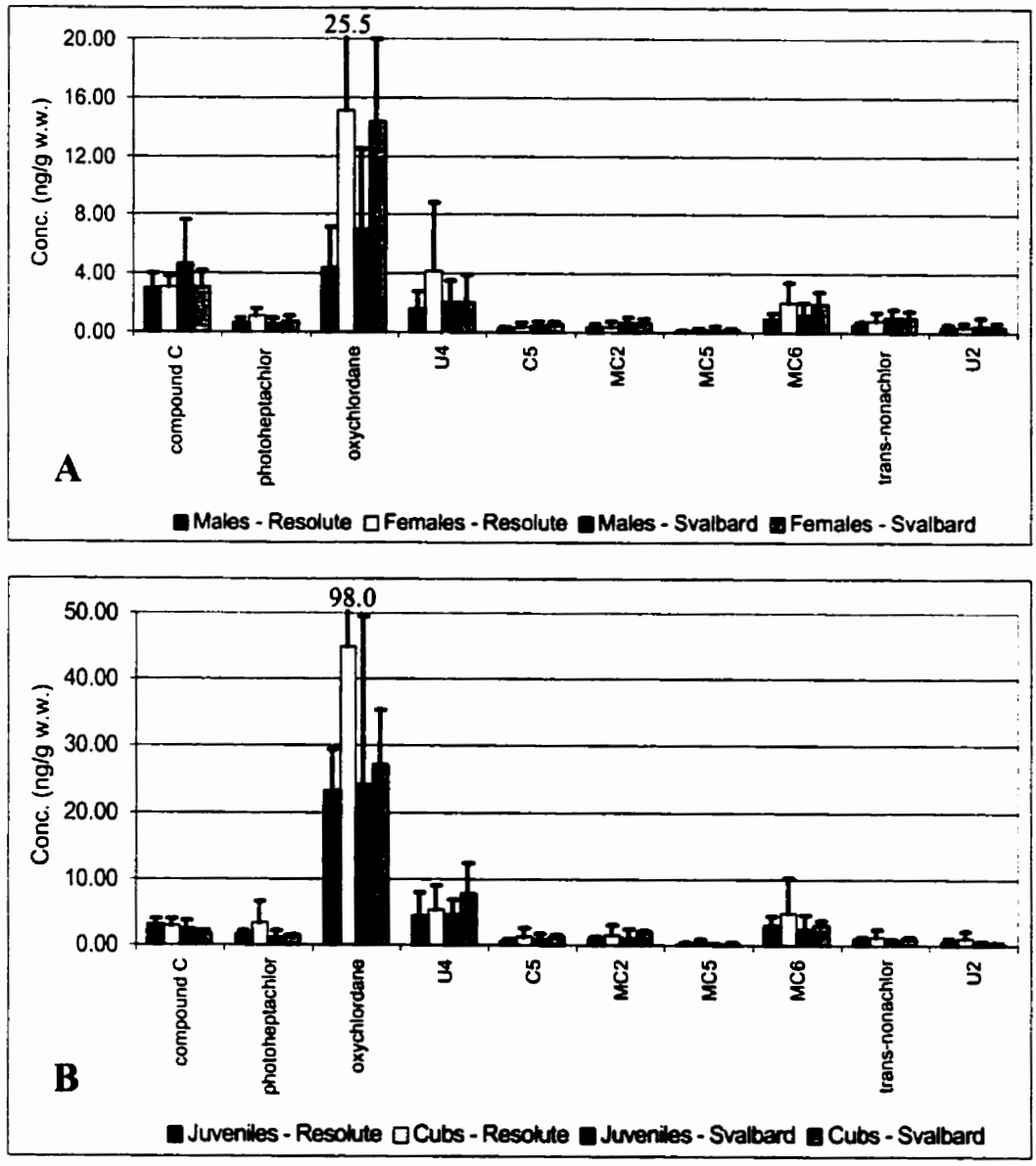


Figure 5.4 - Mean chlordane concentrations (ng/g wet weight) determined in polar bear plasma samples. A - adults only, B - sub-adults (error bars = standard deviation).

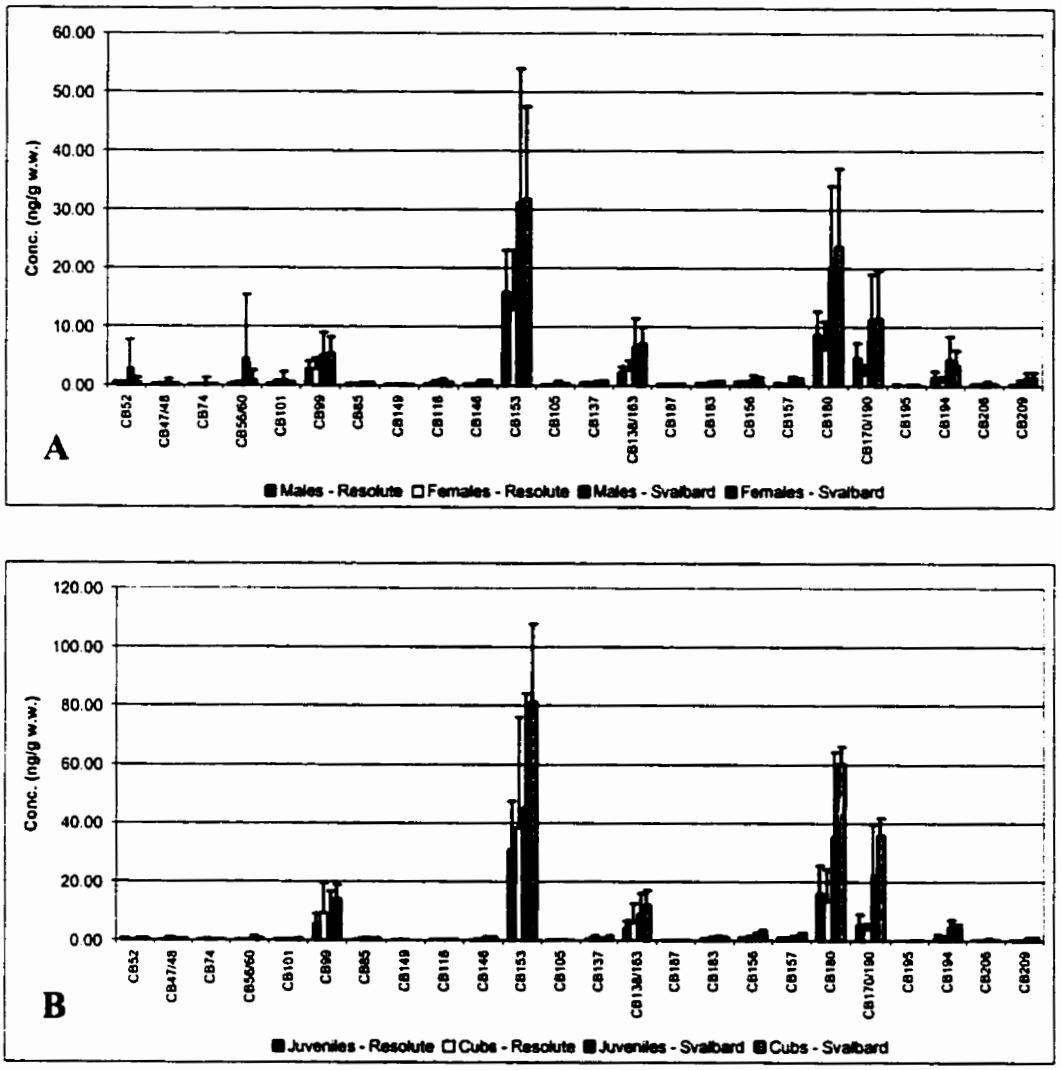


Figure 5.5 - Mean PCB concentrations (ng/g wet weight) determined in polar bear plasma samples. A - adults only, B - sub-adults (error bars = standard deviation).

Ten chlordane compounds were determined in the polar bear plasma samples (Figure 5.4). Chlordane concentrations were similar between regions. There was no significant difference in concentrations of any chlordane compounds analyzed between regions, which is in agreement with previous polar bear data (235) and ringed seal data (63, 264). The main component, oxychlordane, constituted 47% (± 14% SD) of sum chlordanes (ΣCHL = sum 10 chlordane components) in all the samples which is the same mean percentage of sum chlordanes as determined previously (235). Age was negatively correlated with ΣCHL for male bears only ($r=-0.424$, $p=0.02$) and Svalbard bears had a higher correlation coefficient (CC) than Resolute bears. The higher CC has been described previously for the Svalbard population (278). As described by Bernhoft *et al.* (278) for Svalbard bears and Muir and Norstrom (250) for Resolute bears, male polar bears from the present study have lower concentrations of ΣCHL than female bears ($p=0.03$ and $p=0.003$, respectively). It has been hypothesized that lower concentrations and the trend of decreasing concentration with age in males is due to gender differences in metabolizing capacity (278). It was also proposed that since both ΣPCBs and ΣCHL residue levels were found to be related to CYP2B enzyme proteins in polar bear liver (252), higher ΣPCBs in male polar bears induces CYP2B enzymes causing increased metabolism and clearance of chlordane compounds (235). This theory is not supported by Polischuk *et al.* (281) who showed that ΣCHLs were metabolized in male polar bears, but not females, during their fasting period, regardless of contaminant levels. Male-specific metabolism of chlordane compounds may be due to CYP450s other than those induced by contaminants.

PCB residues in polar bears have been extensively studied and concentrations well documented throughout the arctic (235). Twenty-four individual PCB congeners (Figure 5.5) were quantitated. In adults, 15 of the 24 congeners were higher in Svalbard bears (Figure 5.5). Letcher *et al.* (251) found that the ratio of CB99 to CB180 in polar bear adipose decreased with easterly direction from Alaska to East Greenland. The mean ratios of CB99:CB180 in Svalbard and Resolute bears was 0.25 and 0.43, respectively, confirming that higher chlorinated PCBs constituted a higher proportion of the total PCBs in polar bears in the more easterly Svalbard population. Svalbard biota has been shown to have some of the highest concentrations of PCBs in the arctic (235, 265). The proximity of Svalbard to European air and oceanic water flow as well as possible ecological factors may be responsible for the higher Svalbard PCB concentrations.

Male polar bears generally had higher concentrations of PCBs than females but differences were not significant. There seemed to be greater disparity between male and female PCB concentrations in Resolute. The exceptions were the anomalously high CB52 and CB56/60 concentrations in Svalbard males. It is not known why these congeners are so high in the Svalbard male polar bear samples but could possibly be a result of a recent feeding on ringed seals by a few of the Svalbard males. Ringed seals have many lower chlorinated congeners present in their adipose tissue that may cause a sudden surge in lower chlorinated PCBs not normally present at high concentrations in polar bear plasma.

It is concluded that Svalbard bears have higher plasma concentrations of PCBs than Resolute bears ($p>0.0001$, with age and gender as covariates) and that some

gender differences exist (not significantly different). As seen in Figure 5.5, both polar bear cubs and juveniles have higher concentrations of PCBs than adult bears. Lactation is the main source of exposure and is responsible for the cubs having the highest PCB concentration of all age groups (280).

5.3.3. OH-PCBs

Thirty-five compounds in polar bear plasma were identified by mass spectrometry as OH-PCBs. Of these, twenty-four were quantitated using authentic standards or relative response factors of the available authentic standards. The results are sumamrized in Figure 5.6, Table 5.3 and Table 5.4. At the time of this study, there were only 13 authentic OH-PCB standards, however, they constituted the majority of the total. Compounds that have authentic standards were the only congeners used in calculating sum OH-PCBs (ΣOH-PCBs). When all OH-PCBs (including ones without standards) were included in the total, it was referred to as the estimated sum OH-PCBs (est. ΣOH-PCBs). Two other phenolic compounds were quantitated in the phenolic compound fraction, PCP and 4-OH-HpCS (see Chapter 4), are also shown in Figure 5.6.

The highest concentration of OH-PCBs in wildlife or human plasma published to date were those determined in male fish eaters from Latvia (192). In this study, concentrations of 4-OH-CB187 reached a maximum of approximately 4.3 ng/g on a wet weight basis. Mean concentrations of 4-OH-CB187 in adult polar bear plasma were 9 to 12 times higher in Resolute and Svalbard bears respectively than the Latvian

fish eaters. Even though polar bears have been shown to have high $MeSO_2$-PCBs, it was unexpected to find such high concentrations of OH-PCBs in blood.

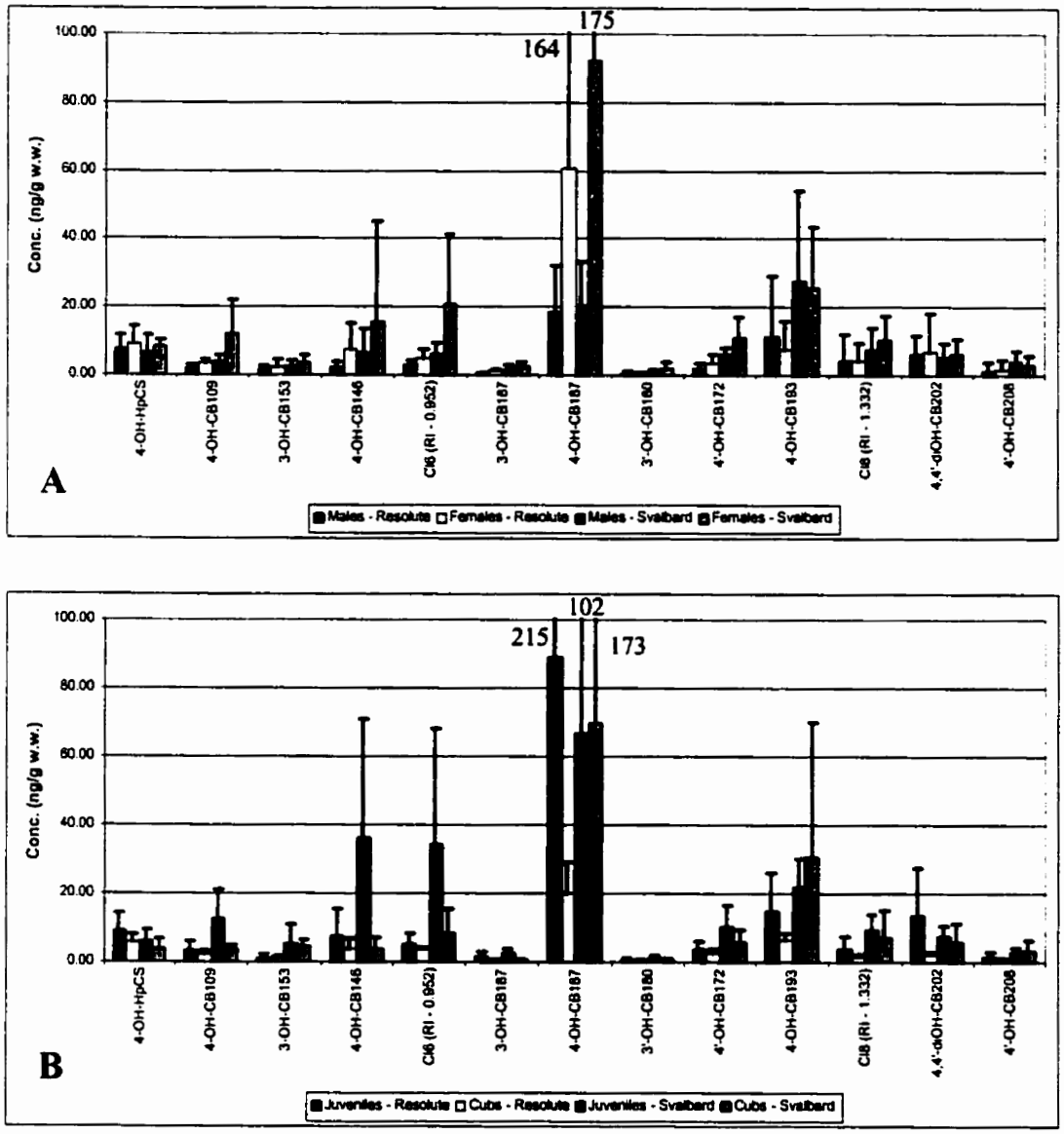


Figure 5.6 - Mean concentrations of selected OH-PCB congeners (ng/g wet weight) determined in polar bear plasma samples. A - adults only, B - sub-adults (error bars = standard deviation).

Table 5.3 - Mean concentrations (ng/g wet weight) of all phenolic compounds quantitated in adult polar bear plasma (SD = standard deviation).

	Resolute Bears				Svalbard Bears			
	Males (n=12)		Females (n=13)		Males (n=18)		Females (n=15)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PCP	0.14	0.06	0.23	0.08	0.14	0.07	0.17	0.10
4-OH-HpCS	7.58	4.04	9.20	5.17	6.58	5.21	8.39	2.00
Cl_5 (RI - 0.601)	0.21	0.15	0.15	0.13	0.29	0.27	0.48	0.65
4'-OH-CB120	0.16	0.12	0.38	0.18	0.31	0.21	0.40	0.24
3'-OH-CB118	0.06	0.09	0.06	0.07	0.04	0.05	0.29	0.33
4-OH-CB109	1.97	1.25	3.33	1.20	3.72	1.83	12.06	10.04
Cl_6 (RI - 0.803)	0.37	0.15	0.53	0.41	0.63	0.43	1.31	1.11
3-OH-CB153	1.42	1.32	2.14	2.24	2.48	1.74	3.49	2.20
4-OH-CB146	2.35	1.42	7.54	7.68	6.39	7.21	15.60	29.56
3'-OH-CB138	0.65	0.45	0.49	0.49	0.92	0.87	2.12	1.76
4'-OH-CB130	0.07	0.07	0.27	0.23	0.22	0.15	0.97	0.95
Cl_6 (RI - 0.952)	2.88	1.12	4.81	2.62	5.93	3.66	20.87	20.42
3-OH-CB187	0.60	0.32	1.08	0.50	1.40	1.52	2.64	1.12
4-OH-CB187	18.48	13.55	60.75	102.86	20.40	13.07	91.92	83.34
Cl_6 (RI - 1.029)	0.30	0.22	0.43	0.51	0.63	0.50	0.99	1.02
4'-OH-CB159	0.37	0.21	0.55	0.38	0.69	0.35	1.30	0.82
Cl_6 (RI - 1.047)	0.78	0.27	0.75	0.61	2.50	1.30	2.34	1.20
Cl_8 (RI - 1.107)	0.98	0.73	1.42	0.60	2.75	1.67	3.36	1.37
3'-OH-CB180	0.51	0.69	0.49	0.35	1.04	0.48	1.95	1.96
4'-OH-CB172	1.99	1.32	3.24	2.64	5.99	1.94	11.02	5.91
4-OH-CB193	11.43	17.64	7.66	8.07	27.55	26.47	25.94	17.73
diOH-Cl_7 (RI - 1.289)	1.00	0.57	1.38	1.50	1.60	0.92	3.03	1.82
Cl_8 (RI - 1.314)	0.41	0.49	0.34	0.29	0.62	0.63	1.16	0.79
Cl_8 (RI - 1.332)	4.08	8.02	3.99	5.55	7.75	6.32	10.25	7.02
4,4'-diOH-CB202	6.00	5.68	6.97	11.06	5.40	4.15	5.89	4.77
4'-OH-CB208	1.29	2.33	1.57	2.82	3.62	3.63	3.19	2.31

Table 5.4 - Mean concentrations (ng/g wet weight) of all phenolic compounds quantitated in sub-adult polar bear plasma (SD = standard deviation).

	Resolute Bears				Svalbard Bears			
	Cubs (n=3)		Juveniles (n=5)		Cubs (n=3)		Juveniles (n=2)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
PCP	0.22	0.10	0.28	0.14	0.07	0.03	0.10	0.01
4-OH-HpCS	5.64	2.44	9.23	4.94	3.73	3.13	5.86	3.45
Cl_5 (RI - 0.601)	0.12	0.08	0.29	0.39	0.18	0.02	1.16	1.07
4'-OH-CB120	0.31	0.16	0.32	0.21	0.23	0.09	0.21	0.02
3'-OH-CB118	0.16	0.05	0.09	0.11	0.09	0.07	0.34	0.27
4-OH-CB109	2.35	1.13	3.35	2.76	3.61	1.45	12.29	9.00
Cl_6 (RI - 0.803)	0.33	0.19	0.73	0.74	0.53	0.20	2.34	1.82
3-OH-CB153	1.27	0.34	1.23	0.93	4.48	1.91	5.29	5.63
4-OH-CB146	3.64	3.27	7.50	8.01	3.72	3.50	36.17	34.71
3'-OH-CB138	0.17	0.09	0.35	0.21	1.60	0.77	1.66	0.33
4'-OH-CB130	0.08	0.03	0.18	0.13	0.34	0.23	1.02	0.84
Cl_6 (RI - 0.952)	3.60	0.56	5.34	3.03	8.30	7.07	34.19	33.96
3-OH-CB187	0.57	0.21	1.52	1.54	0.79	0.07	2.50	1.21
4-OH-CB187	20.00	9.06	89.00	126.37	69.97	103.23	66.80	35.09
Cl_6 (RI - 1.029)	0.73	0.67	0.87	0.74	0.94	0.88	0.72	0.52
4'-OH-CB159	0.99	0.67	1.01	0.72	1.07	0.89	0.98	0.63
Cl_6 (RI - 1.047)	0.53	0.27	1.79	1.49	1.59	0.69	2.71	1.89
Cl_8 (RI - 1.107)	0.74	0.17	1.50	0.82	1.55	0.45	3.22	1.60
3'-OH-CB180	0.38	0.33	0.54	0.69	0.78	0.53	1.30	0.70
4'-OH-CB172	2.70	1.10	3.67	2.33	5.61	3.64	10.31	6.47
4-OH-CB193	6.59	1.78	14.76	11.38	30.39	39.61	21.79	8.52
diOH-Cl_7 (RI - 1.289)	0.54	0.25	2.29	1.62	0.49	0.42	2.89	1.13
Cl_8 (RI - 1.314)	0.16	0.05	0.30	0.37	0.44	0.07	0.65	0.31
Cl_8 (RI - 1.332)	1.60	0.48	3.85	3.77	7.11	7.94	9.35	4.50
4,4'-diOH-CB202	2.39	0.66	13.48	14.12	5.57	5.87	7.51	3.01
4'-OH-CB208	0.64	0.38	1.49	1.59	3.40	2.95	2.89	1.09

Of the 26 compounds measured, only 3 were higher in Resolute bears as compared to Svalbard bears; 4-OH-HpCS, 4-OH-CB120 and 3-OH-CB118. The latter two are minor compounds close to detection limits. The former is a metabolite of OCS that seems to be evenly distributed between both regions. Svalbard bears have on average two times the concentrations of OH-PCBs than Resolute bears. This difference is similar to that observed for PCB levels in the two populations. For adult bears, log transformed ΣOH-PCBs were significantly related to log transformed ΣPCBs ($r=0.29$, $p=0.02$ – not shown). This is a weak relationship compared to a recent human study (151) where PCBs and OH-PCBs were much more highly correlated ($r = 0.84$, $p < 0.005$). The weak correlation in polar bears may be a result of gender, age, or other factors affecting enzyme activity and hence rate of formation of OH-PCBs. Saturation of OH-PCBs binding to plasma proteins may also be occurring.

The latter hypothesis could be tested with additional experimentation by quantitating transport protein concentrations in polar bear blood. Using human blood chemistry values (120) and the mean molar concentration of total phenolic compounds (sum PCP, 4-OH-HpCS and est. ΣOH-PCBs) found in the polar bear plasma, exogenous phenolic compounds would equal 50% of the total carrying capacity of thyroid hormone transport proteins (sum albumin, transthyretin, and thyroxine binding globulin), exceeding the human TTR carrying capacity by 7200%. This suggests that circulating concentrations of chlorinated phenolic compounds may be high enough to affect T4 transport through binding with TTR in polar bear. The uncertainty of this estimate is large but warrants further investigation. Polar bears have been shown to

have only two of the three proteins listed above (147), albumin and TTR, and there are no known publications regarding concentrations of each of the proteins in polar bear blood.

Comparisons of mean ΣOH-PCBs values, showed that juveniles from both regions had some of the highest plasma concentrations of OH-PCBs of the age groups tested (Figure 5.7). Females and cubs also had high OH-PCBs compared to males. Cubs are exposed to PCBs entirely through mother's milk. OH-PCBs are thought to be predominantly bound to TTR and not associated with plasma lipids. They would therefore not distribute to the higher fat content of mother's milk (282). However, there may be some lactational transfer of OH-PCBs since TTR is thought to be present in human milk samples at low concentrations. Some OH-PCBs have been determined in human milk samples (209) and preliminary evidence from our laboratory shows that OH-PCBs are also present in polar bear milk (unpublished results). Since juveniles and cubs are in developmental stages, it would be important to establish concentrations of OH-PCBs in both milk and plasma samples and to further investigate the possible implications of the high OH-PCB exposure for this age category.

Recent evidence indicates that TTR efficiently transports OH-PCBs across the placenta, resulting in fetal levels higher than those of the mother (173). In this same study, 4-OH-CB109 accumulated in fetal brain. Also, fetal total T4 and free T4 levels were reduced by approximately 90% and 40%, respectively when measured at gestational day 20. These results show that OH-PCBs may be affecting circulating thyroid hormone concentrations with possible neurological development implications

since thyroid hormones transported via TTR are the sole source of thyroid hormones to the brain.

Patterns of OH-PCBs in polar bears are similar to those in other species (5, 192), where 4-OH-CB187 was the most dominant congener. The second most abundant congener, 4-OH-CB193, has not been previously determined in biota. The higher chlorinated OH-PCBs, such as 4,4′-diOH-CB202, 4′-OH-CB208 and the unknown OH-Cl_8 compound (RI=1.332), also comprised a large proportion of the total OH-PCBs in both bear populations. The former two congeners have not been identified previously in biota but were mentioned in a recent review by Letcher *et al.* (231). These congeners have only been tentatively identified by their full scan mass spectra (Chapter 2).

Gender differences in the pattern of the main OH-PCB congeners were also observed. When the top 5 congeners were expressed as a percentage of ΣOH-PCBs. The percentage contribution of 4-OH-CB193 to ΣOH-PCBs was significantly higher and 4-OH-CB187 to ΣOH-PCBs was significantly lower in males compared to females ($p<0.001$). When the top 4 congeners were normalized as a ratio of the main metabolite, 4-OH-CB187, male bears had significantly higher 4-OH-CB193 and 4-OH-CB172 ratios than females ($p<0.001$). This is a clear indication of gender differences in OH-PCB congener pattern, which could result from a number of reasons. Reasons may include gender differences in metabolizing enzymes, gender differences in contaminant loads, or both. Further studies are needed to explore and define these trends.

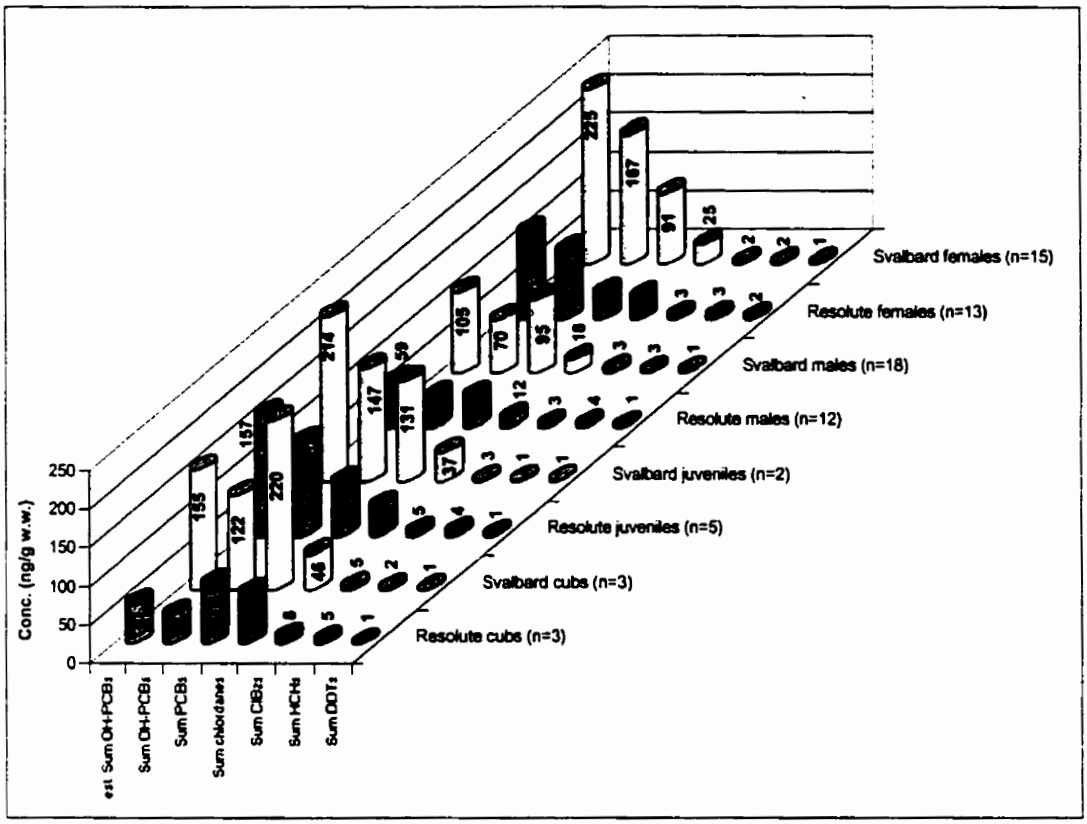


Figure 5.7 - Mean sum concentrations (ng/g wet weight) of contaminant classes in plasma for all age categories, regions and genders of polar bears.

When contaminant classes are compared, OH-PCBs are one of the most important contaminants in polar bear plasma (Figure 5.7). Mean ΣOH-PCBs concentrations were higher than all other chemical classes in most of the bear groups. Mean estimated ΣOH-PCBs were higher than all other classes of compounds in polar bear plasma. In previous OH-PCB studies, the ratio of ΣOH-PCBs to ΣPCBs was always less than 0.30 (151, 191, 192). Adult polar bear ΣOH-PCB: ΣPCB ratios ranged from 0.21 to 6.86 (mean 1.78). This is the first time that OH-PCB concentrations exceeding PCB concentrations has been documented. PCBs were the

second most abundant class of contaminants followed by chlordanes. The other groups of contaminants are considerably less abundant. Therefore, OH-PCBs are a very important class of contaminant to monitor in polar bear plasma as they may possibly display toxicological activity.

Females have higher OH-PCB concentrations and ΣOH-PCB than male polar bears (Figure 5.6 and 5.7). The ratio of ΣOH-PCB: ΣPCB was significantly different between males (mean 1.49) and females (mean 4.08) by Student's t-test ($p < 0.001$). This suggests that either female bears have a higher capacity to slowly metabolize the more persistent PCBs through gender specific enzymes or they have different transthyretin concentrations than male bears which allows larger concentrations of OH-PCBs to be carried in the blood. If the former, the result is contrary to metabolism of chlordane compounds, which is faster in males than females. Further testing is needed to determine the reasons for the higher female OH-PCB concentrations in polar bears.

5.3.4. Principal Component Analysis of Chemical Residue Relationships with Retinol and Thyroid Hormone Concentrations

Many observed effects seen in environmentally exposed animal may not solely be the effect from a single compound or from a single class of compounds. Single classes of compounds may have multiple modes of action depending on structure. For example, mono-ortho PCBs and multi-ortho PCBs can vary dramatically in binding potential to TTR (144), thereby affecting thyroid hormone transport differently. In order to determine relationships using standard analysis of variance procedures, the ratio of samples to parameters should be 5:1. The sample sizes need for this study, which examined over 75 different contaminants, would not be feasible. Principal component analysis (PCA) transforms random or statistical variables into linear combinations that have special properties in terms of variances. It reduces the total number of variables to as many principal components as necessary to maximize the explained variance of the entire data set. The first principal component is the normalized linear combination that explains the highest amount of variance of the data set. The second principal component explains the second most amount of variance and so on. Thus, a large data set can be effectively reduced to a few principal components, which can explain most of the variance of the entire data set consisting of a limited number of samples.

Therefore, PCA was applied to the polar bear chemical residue data and all contaminant groups were included in the analysis for a potential relationship between chemical residues and thyroid hormone and retinol concentrations. Only adults (n=58)

were used in this comparison as sub-adults have elevated levels of contaminants and are in a different life stage that may cause them to have different thyroid hormone and retinol parameters.

PCA was performed using the top 50 contaminants ranked according to concentration. Only compounds quantitated with authentic standards were included, thereby eliminating many of the OH-PCBs.

Using the Scree plot, five eigenvectors were acceptable for the analysis (283), resulting in 72.4% of the total variance explained. The first four eigenvectors were highly ($r^2 > 0.70$) and uniquely correlated with only one of the four classes of compounds ($r^2 < 0.30$ for the other three eigenvectors). The first class of contaminants was persistent PCBs, which loaded highly on the first eigenvector, accounting for 29.1% of the total explained variance [$PC1_{pPCBs}$]. The second class of compounds which loaded highly on the second eigenvector was the OH-PCBs (13.0% variation explained)[$PC2_{OH\text{-}PCBs}$]. The last two classes included the non-persistent PCBs (11.7% explained variance)[$PC3_{npPCBs}$] and chlordane compounds (10.2% explained variance)[$PC4_{CHLOR}$]. Non-persistent PCBs are compounds that are known to be rapidly metabolized by mammals. No particular contaminants loaded onto the fifth eigenvector, which explained another 4.5 % of the variance. As shown previously for chlordane concentrations in male bears, $PC4_{CHLOR}$ were negatively associated with age for both males and females. Since $PC4_{CHLOR}$ were significantly different between males and females, $PC4_{CHLOR}$ was age corrected separately for males and females prior to statistical analysis. None of the other PCs were related to age.

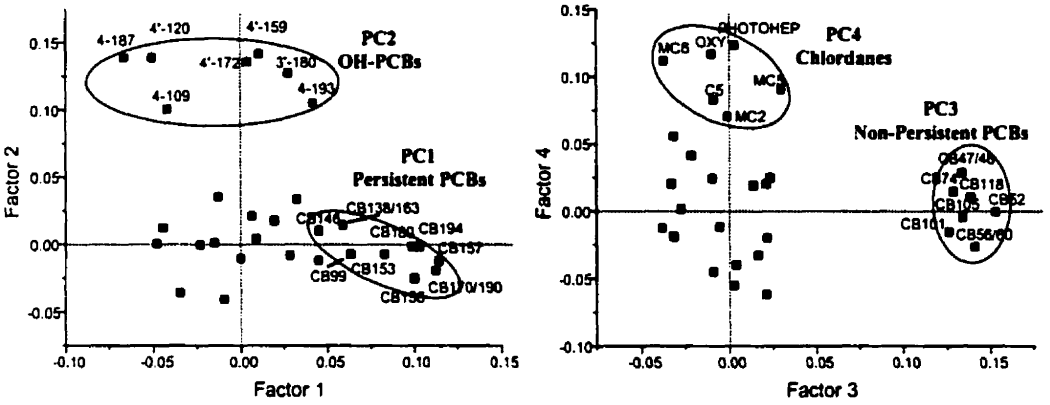


Figure 5.8 - Factor loading plots of the first four principal components, which explain 72.4% of the total variance. Circles were drawn to group contaminants which loaded highly ($r^2 > 0.70$) on each of the top four eigenvectors. All other contaminants loaded less significantly ($r^2 < 0.3$). OH-PCBs are abbreviated by OH-position followed by PCB number.

Table 5.5 shows the top 50 contaminants, correlated factor loading, correlation coefficients and mean adult polar bear population concentrations. As seen from Table 5.5, many of the OH-PCBs rank very high in the total proportion of contaminants. Two more OH-PCBs would also rank in the top 15 if authentic standards were available for quantitation. Factor loading plots for PC1 to PC4 are shown in Figure 5.8. Note that distance away from the origin is proportional to the amount of variance in the whole data set, which is explained by the variable. Contaminants clustering around 0,0 contribute little to the principal components, and were not identified in Figure 5.8. The factor scores of the five principal components were compared to thyroid hormone and retinol concentrations.

Table 5.5 - Top 50 contaminants in adult polar bear plasma ranked by the percentage of the total contaminants. Only compounds with quantitation standards were included in the analysis. The rank and component to which each compound contributed greatest variance (PC) and correlation coefficient (CC) are shown for each compound. The mean concentration of all compounds in each population is also shown. (SD – standard deviation)

Rank	PC	CC	Compound	% Total Contaminants	SD	Mean Conc. Resolute (ng/g w.w.)	Mean Conc. Svalbard (ng/g w.w.)
1	2	0.73	4-OH-CB187	19.11	13.10	24.74	52.91
2	1	0.85	CB153	13.24	5.64	4.81	31.45
3	2	0.70	4-OH-CB193	8.71	7.90	5.79	26.82
4	1	0.92	CB180	8.36	4.55	3.62	21.89
5	4	0.87	oxychlordane	6.70	4.81	9.40	10.36
6	-	-	4-OH-HpCS	4.60	3.26	10.91	7.40
7	1	0.95	CB170/190	4.29	3.11	1.58	11.36
8	-	-	4-OH-CB146	3.73	4.17	2.02	10.58
9	2	0.89	4'-OH-CB172	2.64	1.28	4.20	8.27
10	2	0.71	4-OH-CB109	2.53	1.94	2.42	7.51
11	1	0.79	CB138/163	2.52	1.00	1.35	6.88
12	1	0.77	CB99	2.42	0.98	1.05	5.38
13	-	-	Compound C	2.18	1.53	2.90	3.93
14	-	-	U4	1.74	1.75	0.90	2.08
15	-	-	β-HCH	1.56	1.26	0.54	1.77
16	1	0.74	CB194	1.42	1.29	0.70	4.10
17	4	0.87	MC6	0.94	0.54	1.27	1.55
18	3	0.82	CB56/60	0.81	2.53	0.31	2.97
19	-	-	TeClBz	0.76	0.81	0.47	0.39
20	-	-	HCB	0.75	0.48	0.39	1.88
21	3	0.87	CB52	0.58	1.36	0.34	1.80
22	-	-	DDE	0.57	0.77	0.89	0.90
23	4	0.85	photoheptachlor	0.54	0.38	0.79	0.61
24	-	-	3'-OH-CB138	0.52	0.52	1.89	1.46
25	-	-	*trans*-nonachlor	0.51	0.26	0.54	1.03
26	2	0.73	3'-OH-CB180	0.45	0.34	0.60	1.46
27	1	0.90	CB156	0.43	0.20	0.24	0.99
28	2	0.84	4'-OH-CB159	0.37	0.19	0.28	0.97
29	1	0.96	CB157	0.36	0.22	0.18	0.92
30	4	0.70	MC2	0.33	0.16	0.20	0.63
31	-	-	PnClBz	0.32	0.25	0.29	0.30
32	3	0.76	CB101	0.29	0.40	0.26	0.66
33	4	0.77	C5	0.28	0.13	0.21	0.51
34	-	-	α-HCH	0.26	0.35	0.15	0.58
35	-	-	OCS	0.26	0.20	0.30	0.48
36	-	-	CB137	0.26	0.13	0.23	0.55
37	-	-	U2	0.25	0.23	0.32	0.45
38	3	0.74	CB118	0.25	0.18	0.19	0.52
39	1	0.70	CB146	0.22	0.10	0.20	0.58
40	-	-	CB183	0.22	0.11	0.25	0.47
41	2	0.71	4-OH-CB120	0.19	0.16	0.47	0.35
42	3	0.72	CB47/48	0.18	0.17	0.14	0.45
43	-	-	CB85	0.16	0.12	0.13	0.35
44	2	0.73	4'-OH-CB130	0.16	0.15	0.18	0.56
45	-	-	CB206	0.14	0.12	0.05	0.34
46	3	0.78	CB74	0.13	0.20	0.21	0.30
47	3	0.80	CB105	0.12	0.13	0.09	0.27
48	4	0.70	MC5	0.11	0.10	0.12	0.20
49	-	-	PCP	0.11	0.08	0.23	0.15
50	-	-	CB187	0.11	0.11	0.01	0.20

5.3.4.1. Retinol

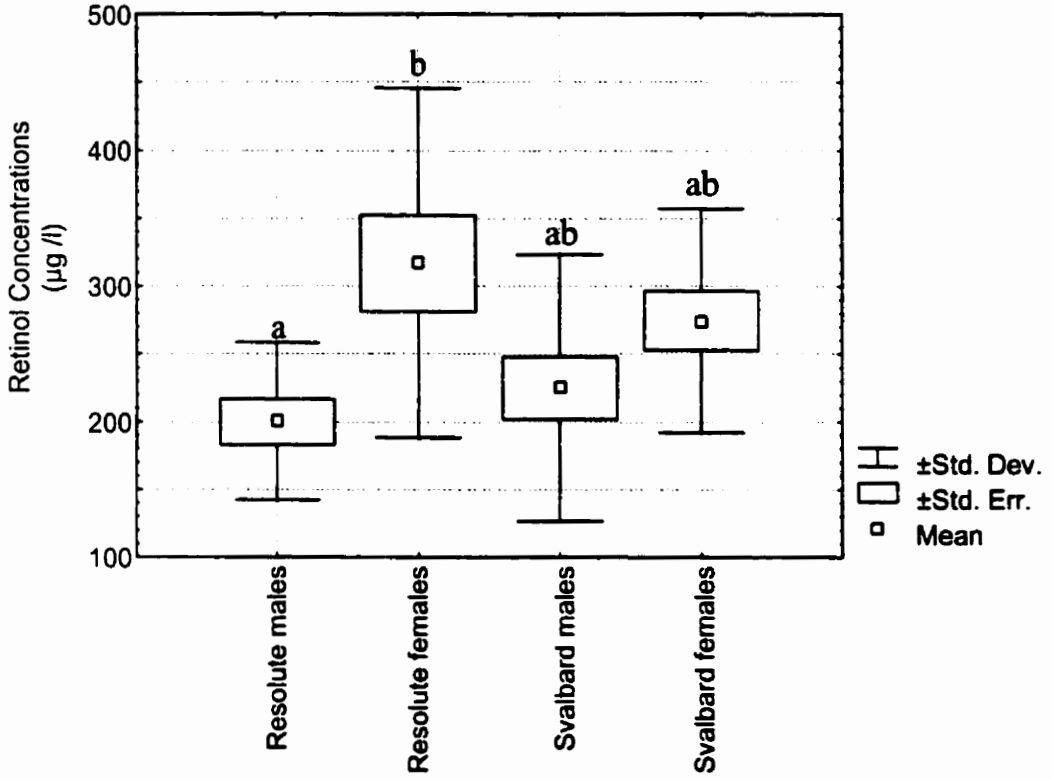


Figure 5.9 - Box plots showing gender differences in polar bear plasma retinol concentrations.

Gender and regional differences in retinol concentrations are shown in Figure 5.9. Only Resolute males and females had statistically different plasma retinol concentrations. When both bear populations were included in the analysis, plasma retinol concentrations were negatively correlated with $PC1_{pPCBs}$ and positively correlated with $PC2_{OH\text{-}PCBs}$ (Figure 5.10). The correlation was largely driven by the data for Resolute bears. When only Resolute bears (n=25) were included in the analysis, $PC1_{pPCBs}$ and $PC2_{OH\text{-}PCBs}$ were correlated with retinol concentrations with correlation coefficients of –0.744 ($p<0.001$) and +0.692 ($p<0.001$). This may

demonstrate that retinol perturbation occurs at relatively low contaminant concentrations in the polar bears since the low exposure group is more highly correlated than the high exposure group. These results are confounded by the fact that Resolute females and males had statistically different ($p=0.008$) retinol concentrations (Figure 5.9), $PC1_{pPCBs}$ scores ($p=0.001$) and $PC2_{OH\text{-}PCBs}$ scores ($p=0.008$) by Student's T-test. When the relationship between principal components and retinol were compared for gender, using only the Resolute population, female retinol concentrations were more highly correlated to $PC1_{pPCBs}$ scores ($r=-0.819$, $n=13$) than males, while male retinol concentrations were dependent on $PC2_{OH\text{-}PCBs}$ scores ($r=0.744$, $n=12$) than females. The sample number limits interpretation and significance of these relationships. Thus, it is not known whether the correlations are a result of the higher PCB concentrations in Resolute males, the higher OH-PCBs in Resolute females or the natural physiological differences between male and female retinol concentrations. In humans, female serum retinol levels were found to be lower than males (284) and in this study, female polar bears had higher levels than males. Thus, no further interpretation of the data is possible until a normal plasma retinol concentration range can be established for the polar bear and larger sample sizes analyzed so gender differences can be corrected prior to statistical analysis. Future studies should include the analysis of additional plasma retinoids so that contaminant effects on retinoid homeostasis may be explored.

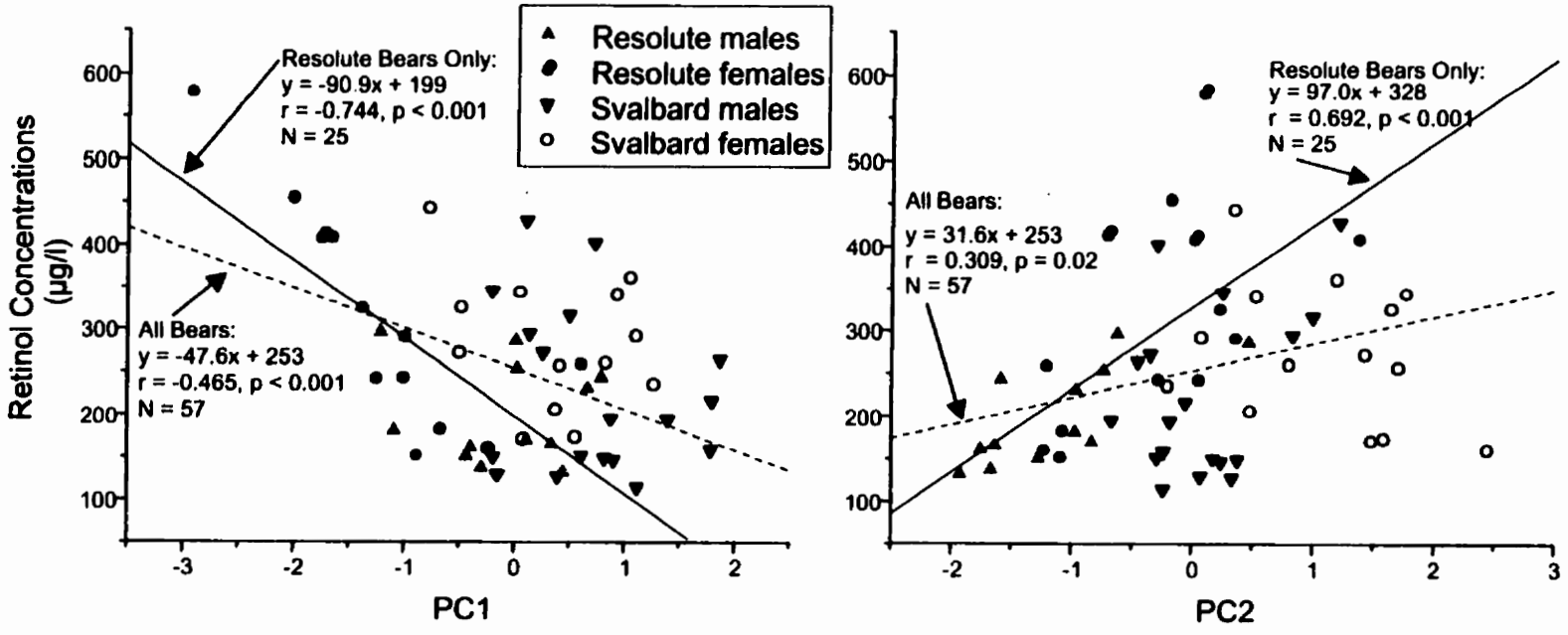


Figure 5.10 - Associations between persistent $PCBs_{(PC1)}$ and $OH\text{-}PCBs_{(PC2)}$ and adult polar bear plasma retinol concentrations (µg/l).

Generally, the negative correlation of $PC1_{pPCBs}$ with plasma retinol follows previous studies where plasma retinol concentrations were shown to decrease with PCB exposure (98, 99, 285). In polar bears, a negative association between log transformed sum PCBs and log transformed plasma retinol concentrations was reported previously (n=57, r=-0.37, p=0.003) (286). The same researchers recently confirmed their results with a significant negative association between log sum PCBs and plasma retinol (r=-0.33, p=0.003) (272). Both of these studies examined Svalbard bears only. Thus, our results are very similar. These studies used total PCBs as a comparative measure to retinol concentrations. The lower correlation coefficient seen in these studies as compared to the association between $PC1_{pPCBs}$ and retinol in the present study may be due to the non-persistent PCBs. Skaare *et al.* (272) showed that non-persistent PCBs, such as CB105 and CB118, were not negatively associated with plasma retinol concentrations in Svalbard bears which is in agreement with our findings where $PC3_{npPCBs}$ scores were not significantly associated with plasma retinol.

The association between $PC2_{OH\text{-}PCBs}$ and plasma retinol in this study is not easily explained. The anticipated relationship between OH-PCBs and plasma retinol would be a negative association due to interference of OH-PCBs and the transport of retinol via RBP:TTR dimer formation. The mechanism behind the positive correlation between retinol and $PC2_{OH\text{-}PCBs}$ in this polar bear study can be clarified with discussion of thyroid hormone measurements.

5.3.4.2. Thyroid hormone measures

Measurement of plasma T4 and T3 concentrations and determination of free T3 and T4 indices assessed effects of CHCs on thyroid hormone homeostasis. The

latter thyroid hormone measurements have been shown to be an accurate indirect measure of percentage free hormone concentrations, as direct measurements of free hormone concentrations are difficult (277). Since the free T4 index was chosen over measuring free T4 concentrations, we were unable to compare our thyroid hormone results with a recent publication, where the total T4 to free T4 ratio was found to be negatively associated with ΣPCBs (r=-0.28, p=0.01) in polar bears (272).

Total T4 plasma concentrations were negatively associated with both $PC1_{pPCBs}$ (r=-0.337, p=0.01, n=56) and $PC3_{npPCBs}$ (r=-0.293, p=0.03, n=56), but not with any other contaminant group, including OH-PCBs. Since both principal components are PCB related, this suggests a common mechanism of action of all PCB congeners in reduction of plasma T4 concentrations. Total T4 concentrations were also negatively correlated with concentration of ΣPCBs (r=-0.29, p=0.04, n=56). PCBs have been shown to reduce total T4 plasma concentrations (287) and it is thought that increased peripheral T4 metabolism is responsible for decreased plasma levels (162). A previous polar bear study was unable to determine statistically significant relationships between ΣPCBs and T4 concentrations (272).

T4 concentrations in plasma are usually assumed to be related to OH-PCBs at the thyroid hormone transport protein level since many OH-PCBs have been shown to bind with TTR with quite high affinity (58, 142). The free thyroid hormone indices are relative measures of the binding potential of the plasma for the thyroid hormones, which were correlated to the percentage of free thyroid hormones in some species (277). A low index value is indicative of a high binding potential of the plasma for that particular thyroid hormone. When the free thyroid hormone indices were

analyzed for correlations to the main PCs, only $PC2_{OH\text{-}PCBs}$ were found to be significantly, negatively correlated (Figure 5.11) with the free T4 index using the whole data set. Individual OH-PCB congeners and ΣOH-PCBs were negatively associated (not significantly) with the free T4 index. When separated by gender, only male bears showed significant correlation between $PC2_{OH\text{-}PCBs}$ and the free T4 index. When separated by region, $PC2_{OH\text{-}PCBs}$ were no longer correlated to the free T4 index. In Svalbard samples, $PC1_{pPCBs}$ were correlated with the free T4 index ($r=0.40$, $p=0.004$, $n=44$). No PCs were related to the free T4 index for the Resolute population.

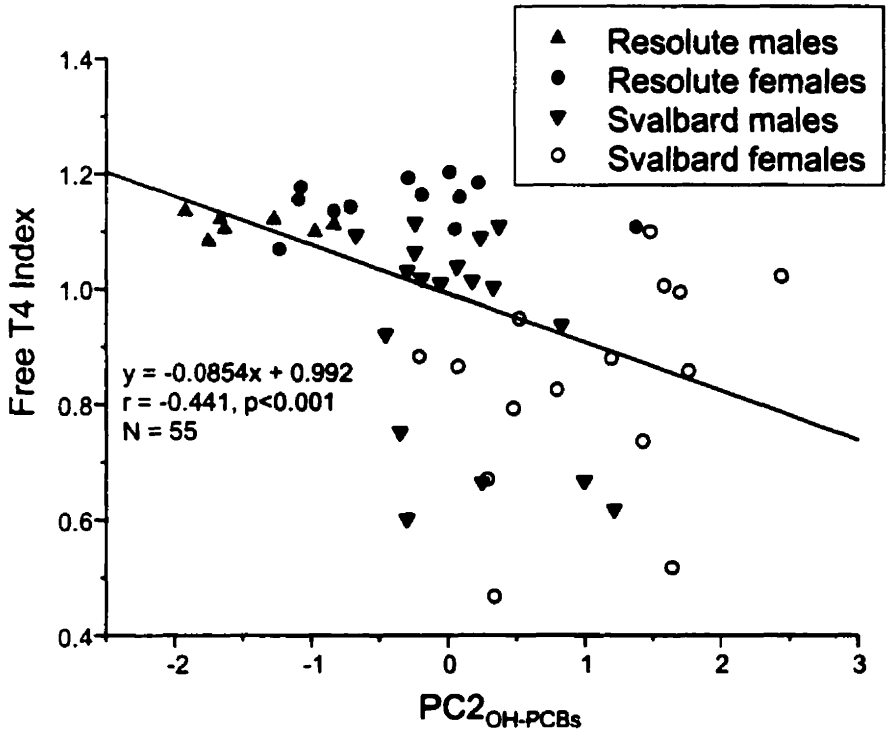


Figure 5.11 - Significant negative association between free T4 index of polar bear plasma and $PC2_{OH\text{-}PCBs}$.

The negative correlation of $PC2_{OH\text{-}PCBs}$ and the free T4 index demonstrates that increasing OH-PCB concentrations are associated with a decreasing free T4 index, or higher plasma binding potential of the plasma. It is not known why the plasma T4

binding potential is higher in the Svalbard bears, but the higher T4 binding potential may be responsible for the retention of more OH-PCBs, resulting in elevated concentrations of OH-PCBs in the Svalbard population. Both populations (high and low exposures) were needed to obtain a significant relationship. The variation in the free T4 index was shown to be different between populations (Figure 5.12). This might be interpreted as the lower exposure Resolute bears having a smaller variation in T4 binding potential and represents the "normal range" of the T4 binding index for polar bears. The variation in the free T4 binding index for the Svalbard bears is much larger. The larger variation in the free T4 binding index may be responsible for the larger variation in the OH-PCB concentrations seen in the Svalbard population.

The observation of larger variation in biochemical parameters in high exposure test subjects has not been documented before but studies on thyroid hormone concentrations in fish exposed to chlorinated pesticides have shown similar trends in the variation of the data sets. The data shows that exposed populations seem to have greater variation in biological measures compared to controls used in the same experiment (288).

However, there is still a significant inverse relationship for the free T4 binding capacity of the plasma and OH-PCB exposure. This is an important finding since the relationship may be responsible for the higher OH-PCB concentrations seen in the Svalbard population.(142, 173) This preliminary evidence should be further explored by equilibrium dialysis to determine which transport proteins are higher in the Svalbard bears resulting in their increased T4 binding capability. If TTR concentrations are found to be higher in Svalbard bears compared to Resolute bears,

then a mechanism exists for enhanced retention of OH-PCBs resulting in higher plasma OH-PCB concentrations. The reasons for the enhanced T4 binding capabilty in the Svalbard bears also needs to be determined since it is not known whether it is contaminant related.

When TTR is secreted into the blood stream from the liver, it is usually co-secreted as a dimer with retinol binding protein. If OH-PCBs are positively associated with T4 binding capability and if T4 binding capability is increasing due to higher TTR concentrations, then it is possible that OH-PCBs would be positively associated with retinol concentrations (shown in Figure 5.10). The evidence for this relationship is circumstantial and needs further experiments to elucidate the mechanisms behind the observed relationships.

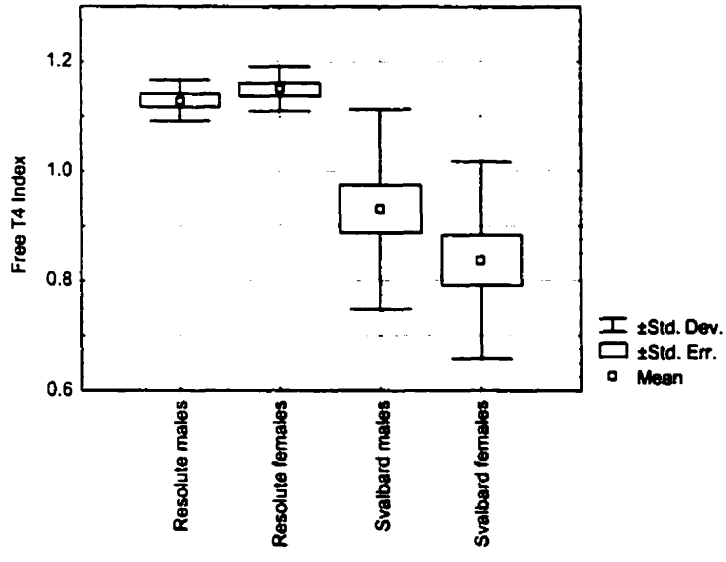


Figure 5.12 - Box plots showing regional differences in T4 binding capacity of polar bear plasma. Figure demonstrates the large variation in the high exposure group (Svalbard bears) as compared to the low variation in the low exposure group (Resolute Bears).

5.4. Conclusions

Polar bear plasma was found to have the highest OH-PCB concentrations ever determined in biota, with mean levels approximately 10 times greater than the highest recorded human exposure. Polar bears are the first species to demonstrate OH-PCB concentrations, which exceeded PCBs and all other CHCs. The two populations of polar bears in this study represented the broad levels of contamination necessary for comparing chemical residues to biochemical measures. Unfortunately, gender may have confounded results so that relationships were not statistically significant due to genders having to be analyzed separately resulting in insufficient sample sizes for the analysis. Using principal component analysis, it was found that PCBs were negatively correlated to retinol concentrations thus indicating a possible decrease on polar bears' retinol levels with PCB exposure. Even though OH-PCBs were positively correlated with PCBs, OH-PCBs were positively correlated to retinol concentrations for reasons that are uncertain at this time. OH-PCBs were determined to be negatively correlated to the the free T4 index for polar bear plasma possibly explaining the higher concentration of OH-PCBs found in the Svalbard polar bear population.

Due to the high levels of OH-PCBs found in polar bear plasma and their potential for endocrine disruption, it is important to examine this class of contaminants in future polar bear studies.

5.5. Acknowledgements

The author acknowledges Environment Canada and the National Wildlife Research Center for their support of this research. This study was funded by the Northern Contaminants Program, Department of Indian and Northern Affairs. Mitra Brown and Scott Brown (Canadian Center for Inland Waters (CCIW), Burlington, ON, Canada determined thyroid hormone measurements. The author would like to thank Suzanne Trudeau, Kim Williams and France Maisonneuve for their help in the analysis of plasma retinol. I would also like to thank Jason Duffe for his assistance in the statistical analysis of the data and Sue Newson for her technical assistance. I would like to dedicate this chapter to the late Dr. Malcolm Ramsey. Polar bear research will not be the same without him.

Chapter 6. Analysis of hydroxylated metabolites of PCBs (OH-PCBs) and other chlorinated phenolic compounds in whole blood from Canadian Inuit [‡]

6.1. Background

Polychlorinated biphenyls (PCBs) have been extensively studied since their discovery as environmental pollutants over 30 years ago (289). Their persistence in biota is well known but the mechanism of their adverse effects on biological systems is still not completely understood. Some of their toxicity may be linked to the biotransformation products of PCBs (231). PCBs are biotransformed by a diverse enzyme system, the cytochrome P450 monooxygenases. Most of the known metabolic pathways involve the initial formation of hydroxylated metabolites. Even one of the more recalcitrant PCBs, CB153, has been shown to be metabolized *in vitro* and *in vivo* to a number of phenolic metabolites (6, 51, 53, 290). Phenolic metabolites can be excreted unchanged, as they were first discovered in the excreta of Baltic seals and Guillemots (200), or further conjugated with glucuronic acid or sulfate (291). The introduction of a hydroxyl group increases the polarity of the PCB and facilitates excretion. If the hydroxyl group is *para* to the phenyl-phenyl bond and has adjacent chlorine atoms, the structure resembles the prohormone, thyroxine (T4) (246). This structural similarity allows OH-PCBs to bind with high affinity to one of the thyroid hormone transport proteins, transthyretin (TTR) (58, 172, 258, 292). For example, 4-

---

[‡] Adapted from C.D. Sandau, P. Ayotte, É. Dewailly, J. Duffe, R.J. Norstrom, **2000**. *Environ. Health Perspect.* 108(7):611-616.

OH-3,3′,4,5′-tetrachlorobiphenyl, a metabolite of CB77, has a binding affinity to TTR four times stronger than T4 (292) and has been shown to disrupt thyroid hormone and retinol transport (102). This is the presumed mechanism by which OH-PCBs are selectively retained in plasma and a possible mechanism of PCB toxicity (5). This type of interaction is not limited to OH-PCBs. Pentachlorophenol (PCP) binds with two times the affinity of T4 to TTR (72), indicating that other chlorinated phenolic compounds may also be interfering in thyroid hormone transport.

Some Inuit consume traditional foodstuffs which may consist of fatty tissues from sea mammal species such as ringed seal and beluga (293-295). Ringed seal blubber, beluga skin and beluga blubber in northern Canada contain average total PCB concentrations of 1,283, 145 and 5,000 ng/g wet weight, respectively (296, 297), which is much higher than that in the diet of the general population (298). Thus, the Inuit population may be exposed to large doses of PCBs. In one study, mean PCB blood levels in Inuit were 30 times those of a southern population (299). An increased PCB body burden could result in the increased formation of metabolites due to induction of the P450 system. Increased levels of metabolites may be significant enough to disrupt thyroid hormone transport. Alterations of thyroid hormone status have been proposed as a mechanism of action by which PCBs would induce adverse neurodevelopmental effects (300). It is therefore of interest to study the concentrations of OH-PCB metabolites in the Inuit population as a first step to evaluate their possible implications mediating PCB-induced health effects. Hydroxylated PCBs have never been studied in the Arctic environment and very little is known about levels and patterns in humans. There are only two other published

studies on OH-PCB metabolite concentrations in human blood from Sweden and Latvia (5, 192).

6.2. Materials and Methods

During the Fall of 1992, 499 Inuit adults living in Nunavik (Northern Quebec) participated in the *Santé Quebec Health Survey*. After signing an informed consent form, a 30 ml blood sample was drawn by venous puncture for organochlorine determination. Thirty sub-samples were randomly selected out of these 499 samples for chlorinated phenolic compound and additional PCB residue analysis. The *Laval University Medical Centre* (Sainte-Foy, Quebec) donated a southern Quebec general population sample of pooled whole blood for comparison to the Inuit population.

The following $^{13}C_{12}$ labeled standards acquired from Wellington Laboratories (Guelph, ON, Canada) were used as recovery internal standards for OH-PCB determination: 4′-OH-CB120, 4′-OH-CB159, 4′-OH-CB172 and 4-OH-CB187. PCP ($^{13}C_6$) was purchased from Cambridge Isotope Laboratories (Andover, MA) and used for PCP quantitation. A synthesized performance standard, 4′-Me-4-MeO-2,3,3′,5,6-pentachlorobiphenyl, was added to all phenolic fractions prior to mass spectral analysis. Labeled PCBs ($^{13}C_{12}$ - CB28, 52, 118, 153, 180 and 194) were used as recovery standards and $^{13}C_{12}$-CB138 as the performance standard for PCB analysis by the external standard method. The $^{13}C_{12}$-PCB standards were purchased from Cambridge Isotope Laboratories (Andover, MA).

Whole blood samples (mass range from 1.54 to 5.76 g) were spiked with $^{13}C_{12}$ recovery standards prior to extraction. The standards consisted of a PCB mixture

($^{13}C_{12}$ - CB-28, 52, 118, 153, 180 and 194, 10 µl, 2.5 ng/µl), an OH-PCB mixture ($^{13}C_{12}$ - 4'-OH-CB120, 4'-OH-CB159, 4'-OH-CB172 and 4-OH-CB187, 20 µl, 100 pg/µl) and PCP ($^{13}C_6$ – 100 µl, 100 pg/µl). The samples were then extracted using a similar method to that described in Chapter 3. The organic phase from the potassium hydroxide partitioning, which contains the neutrals, was reduced in volume and applied to a Florisil column (8 g, 1.2% deactivated). The PCBs were collected in one fraction of 75 ml dichloromethane (DCM):hexanes (1:1), reduced in volume and applied to a silica/sulfuric acid (3 g, 22%) column to remove co-extracted biogenic components. The PCBs were eluted in 50 ml DCM:hexanes, reduced to final volume (50 µl) by rotoevaporation and spiked with performance standard ($^{13}C_{12}$ – CB138, 5 µl, 2.0 ng/µl) for mass spectral analysis.

The partitioned phenolic compounds were acidified and back extracted with hexanes, dried over sodium sulfate and derivatized with diazomethane. Diazomethane was generated as needed from nitrosomethylurea precursor as described in Chapter 2. The derivatized compounds were cleaned up on a silica/sulfuric acid (5 g, 22%) column and eluted with 50 ml DCM:hexanes. Samples were reduced in volume by rotoevaporation and brought down to final volume (25 µl) by a gentle stream of nitrogen. All samples were then spiked with a performance standard (4'-Me-4-MeO-2,3,3',5,6-pentachlorobiphenyl, 5 µl, 200 pg/µl) prior to analysis.

Analyses were completed on a Hewlett Packard (Atlanta, GA) 5890A Series II gas chromatograph equipped with an HP 7673A automatic injector and a Hewlett Packard 5988A mass spectrometer. Helium was used as carrier gas and the head pressure was set at 80 kPa. All injections (2 µl) were made in splitless mode onto a

DB-5 ([5%-phenyl]-methylpolysiloxane - 30m x 0.25mm i.d., 0.25 μm film thickness; J&W Scientific Inc., Folsom, CA.) column. Injector temperature was set at 250°C. For the phenolic compound fraction, gas chromatography and mass spectrometry were completed as described in Chapter 3. The phenolic compound fraction was analyzed using ECNI due to the presence of residual biogenic material that interferes with the analysis in electron impact (EI) mode. The PCB fraction is free of biogenic contamination allowing them to be analyzed using selected ion monitoring in EI mode. The electron energy was 70 eV and the source temperature was 200°C. The GC temperature program for the PCB analysis was 100°C for 3 minutes, 10°C/min to 180°C, and then 2.5°C/min to 280°C.

Because analysis was carried out on methylated OH-PCBs, a standard mixture of MeO-PCBs: [4′-MeO-CB120, 4′-MeO-CB107, 4-MeO-CB188, 3-MeO-CB153, 4-MeO-CB146, 3′-MeO-CB138, 4′-MeO-CB130, 3′-MeO-CB187, 4′-MeO-CB175, 4′-MeO-CB159, 3′-MeO-CB180, 4′-MeO-CB172 and 4-MeO-CB193] supplied by Dr. Åke Bergman (Wallenberg Laboratories, Stockholm, Sweden), synthesized as described elsewhere (211), was used for quantitating MeO-PCBs by the external standard method.

A serial dilution of the MeO-PCB mixture was used for quantitation. Response factors relative to the performance standard (relative response factors, RRFs) for each of the compounds in the standard mixture were generated. The RRFs were used to quantitate all identified and unidentified OH-PCBs in each sample by the external standard method, with volume correction using the performance standard. Identified compounds were quantitated using their RRF. Structures were considered

confirmed if they had identical spectra and matching retention times on three GC columns with varying polarities (DB5, DB1701, and DB210 from J&W Scientific Inc., Folsom, CA) as described in Chapter 2. Unidentified compounds were characterized by fullscan mass spectrometry to determine the chlorination pattern. For these compounds, the average RRFs for that chlorination pattern was used for quantitation. The RRFs for compounds with a given chlorination and methoxy group substitution were comparable. For example, the RRFs for the para-methoxylated heptachlorobiphenyls were all within 10% of the average RRF. Recoveries were determined using the $^{13}C_{12}$ OH-PCB standards and 4′-Me-4-MeO-2,3,3′,5,6-pentachlorobiphenyl as the performance standard. PCP was quantitated by isotope dilution using the $^{13}C_6$ internal recovery standard correcting for the 3.4% contribution of native PCP (m/z = 280 amu) to the main ion of the $^{13}C_6$ standard (m/z = 286 amu) cluster.

PCBs were quantitated on a congener-specific basis by the external standard method using an Aroclor 1242:1254:1260 (1:1:1) secondary quantitation standard solution calibrated against primary standard PCB congener solutions provided by the National Research Council of Canada (Marine Analytical Chemistry Standards Program, Halifax, NS, Canada). Concentrations of congeners, for which standard solutions were not available, were determined in the Aroclor mixture by GC using flame ionization detection (235). Aroclors were purchased from Monsanto (St. Louis, MO).

PCBs have been determined previously in these samples by the *Quebec Toxicology Centre* (Sainte-Foy, Quebec) using plasma as the tissue for analysis. Due

to Aroclor 1254 contamination in one of our rotoevaporators, fifteen of the thirty whole blood samples were irreclaimable for PCB quantitation. The fifteen whole blood samples not contaminated with Aroclor 1254 were compared to the previous plasma analyses. Concentrations of all previously quantitated congeners (CB28, CB52, CB99, CB101, CB105, CB118, CB128, CB138, CB153, CB156, CB170, CB180, CB183, CB187) and sum of congeners in plasma were highly correlated ($r > 0.97$) to the whole blood concentrations using least squares regression analysis. The regression analysis for CB153 and total PCBs are given as examples in Figure 6.1. Thus, the previous PCB plasma concentrations for the fifteen samples not quantitated in our lab were adjusted by applying a correction factor from the regression analysis. The adjusted PCB concentrations and the values determined in the fifteen whole blood samples were used in the present study for comparison to OH-PCB concentrations and for subsequent statistical analysis. Aroclor contamination had no effect on the quantitation of OH-PCBs in the contaminated samples since PCBs elute earlier and have no fragment ions that interfere with OH-PCBs detection.

All solvents were residue analysis grade and purchased from EM Science (Gibbstown, NJ, USA). Florisil (Pesticide Analysis Residue grade, 60-100 mesh) was purchased from BDH Inc. (Toronto, ON, Canada). Merck Silica gel (Grade 60, 70-230 mesh, 60A) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Sulfuric acid (Trace metal grade) was purchased from Fisher Scientific (Pittsburgh, PA).

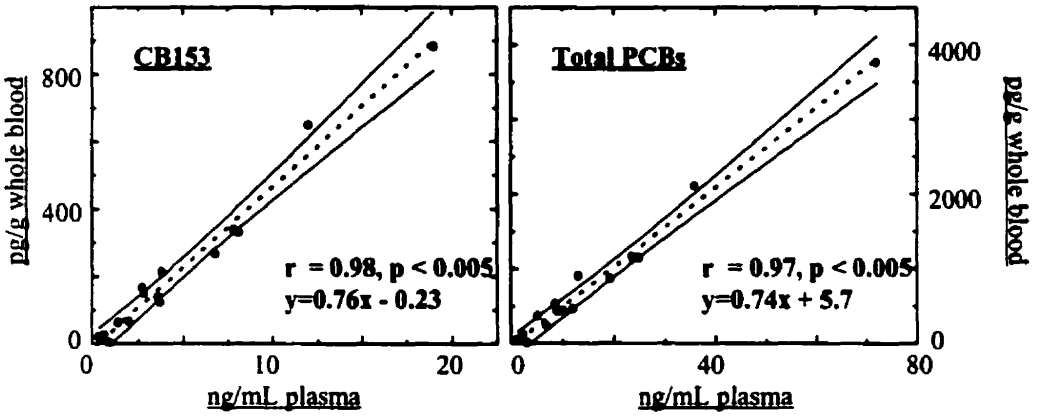


Figure 6.1 - The regression analysis of CB153 and total PCBs that shows that the concentrations previously determined in plasma are significantly correlated to those in whole blood determined in this study. The correction factor was developed by the regression of the whole blood concentration versus the plasma concentration from the previous analyses. The equation for the least squared regression can then be used to adjust the plasma values for the samples into whole blood equivalents. This was done for each congener independently and for the sum of all quantitated PCBs.

6.3. Results

Whole blood recoveries of OH-PCBs were variable, ranging from 50-105%. The mean recoveries for $^{13}C_{12}$- 4'-OH-CB120, 4'-OH-CB159, 4'-OH-CB172 and 4-OH-CB187 were 82%, 69%, 72% and 76%, respectively. This was the first study to use $^{13}C_{12}$ labeled OH-PCBs for accurate OH-PCB quantitation. PCB recoveries had a slightly lower mean recovery of 60%. All PCB and OH-PCB concentrations were consequently recovery corrected.

An example of a chlorinated phenolic ECNI-MS fullscan chromatogram is given in Figure 6.2. The major peaks are identified when known. The OH-PCB fraction in Inuit whole blood contained over 30 congeners, of which 11 were positively identified with authentic standards. Positive identification is based on identical mass spectra using fullscan ECNI mass spectrometry and matching retention

times using three GC columns with varying polarities (results not shown). All identified congeners are listed in Table 6.1. They constituted between 59-81% of the total OH-PCBs in the samples (mean of 70%).

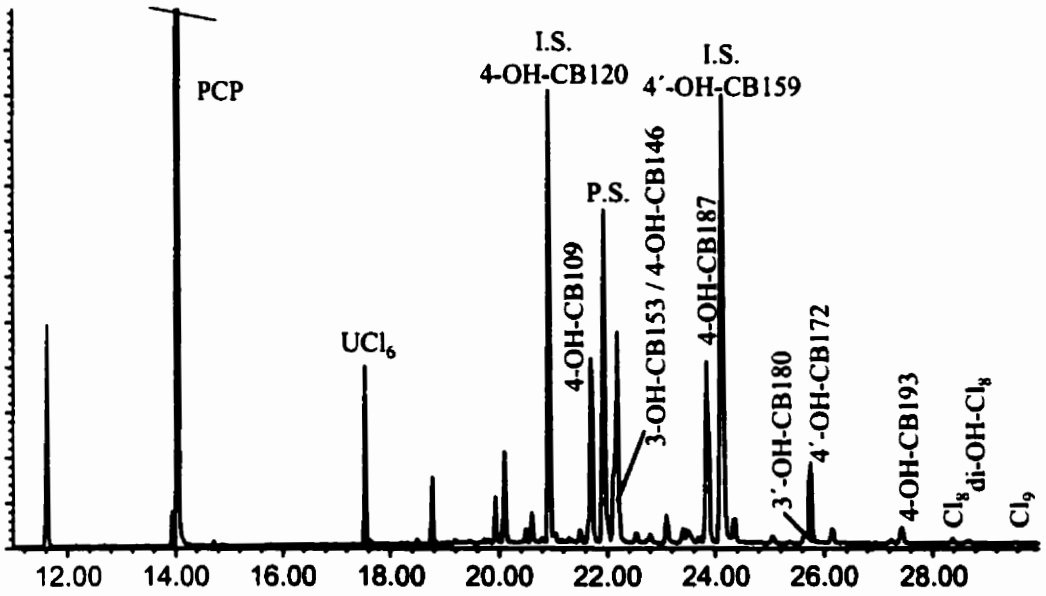


Figure 6.2 - GC-MS(ECNI) SIM chromatogram of the OH-PCBs and other chlorinated phenolic compounds from Inuit whole blood analysis. PCP has been truncated since it is too large to fit on the scale shown. The chromatogram can only be used as qualitative graph since each compound fragments differently and response varies for compounds with different chlorination patterns. For this sample, only two internal standards (I.S.) and the performance standard (P.S.) where added for illustrative purposes.

Table 6.1 - Concentration of PCP and hydroxylated metabolites (ng/g whole blood wet weight x 10^3) in Inuit whole blood. Total OH-PCBs, sum five main congeners (*) and sum identified congeners are given for comparison with previous studies.

	Males (n=13)			Females (n=17)			Southern Population Pool
	Geometric Mean	Range min	Range max	Geometric Mean	Range min	Range max	
Age	38	18	66	38	18	72	-
PCP	2740	1350	7770	1590	558	7510	6290
4'-OH-CB120	12	3	67	7	1	39	3
4-OH-CB109 (*)	314	39	2570	234	15	1470	25
3-OH-CB153 (*)	85	5	537	49	4	830	2
4-OH-CB146 (*)	219	10	1750	111	10	1880	8
3'-OH-CB138	37	3	225	20	2	401	2
4'-OH-CB130	15	1	506	4	<1	47	<1
3'-OH-CB187	17	1	213	11	<1	167	1
4-OH-CB187 (*)	293	22	1840	152	26	2260	31
3'-OH-CB180	17	2	134	8	!	239	n.d.
4'-OH-CB172 (*)	74	5	443	38	4	740	11
4-OH-CB193	41	3	659	26	2	95	18
Sum (*) congeners	1040	88	6740	614	59	7070	76
Sum identified	1210	119	7520	712	87	8060	81
Sum all OH-PCBs	1730	162	10100	1010	17	11600	161
Sum PCBs	1290	2070	65900	7940	1199	38100	488
CB-153	3120	460	13900	1960	263	9510	74

The lipid content of the whole blood samples was not determined. Therefore, all concentrations hereafter are expressed on a whole blood wet weight basis. The chemical residue data was not normally distributed using Liliefors test for normal distribution. Log transformed data approached normal distribution, therefore log-transformed data was used in all statistical analyses and geometric mean values were used unless otherwise stated. The geometric mean concentrations in men and women of all identified congeners and their range of values are listed in Table 6.1. Women

consistently had lower geometric mean concentrations of all the chlorinated phenolic compounds quantitated. Only for PCP was the difference statistically significant between men and women using Student's t-test.

The concentrations in the pooled sample are equivalent to arithmetic mean concentrations. The arithmetic mean of the total OH-PCBs in Inuit whole blood was 1.89 times greater than the geometric mean. Assuming that the distribution of concentrations was similar in the two populations, the geometric mean concentration in samples, which make up the pool would be lower than that indicated in Table 6.1. Therefore the true difference in means is probably underestimated by the data in Table 6.1.

The main chlorinated phenolic compound in the Inuit whole blood was PCP. It contributed between 14-89% of the total chlorinated phenolic compounds quantitated. The concentration of PCP ranged from 0.558 to 7.77 ng/g with a geometric mean of 2.02 ng/g wet weight. The pooled whole blood sample from the southern population had higher PCP levels than all but two Inuit samples analyzed with a concentration of 6.29 ng/g. PCP composed 97% of the total quantitated chlorinated phenolic compounds in the southern population.

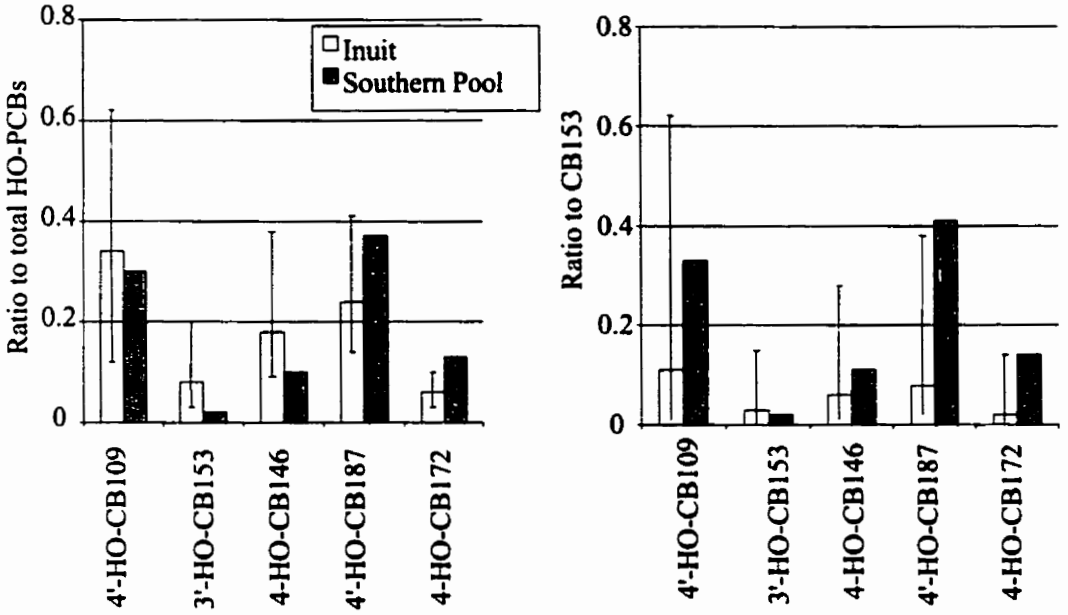


Figure 6.3 - Ratio of the five main metabolites to total OH-PCBs and to CB153. Mean ratios are given for combined male and female Inuit samples and error bars represent the range.

The major OH-PCB congeners varied considerably among individuals. The mean ratios of the five main congeners to total OH-PCBs are shown in Figure 6.3 with bars representing the range. The main congener identified in Inuit whole blood (21 of 30 samples) was 4-OH-CB109 with a geometric mean concentration of 0.266 ng/g and a range from 0.015 to 2.55 ng/g. This congener is probably a mixture of two congeners, 4-OH-CB109 (4-OH-2,3,3′,4′,5-pentachlorobiphenyl) and 4′-OH-CB107 since they co-elute and separation was not achieved with the DB-5 column used in this study. It should be noted that this peak was quantitated using only 4′-OH-CB107 as the quantitation standard since 4-OH-CB109 was not commercially available. The peak is assumed to be predominantly 4-OH-CB109 as it was previously determined to be the predominant compound of the mixture in human plasma at a five to one excess

(5, 201). However, considering pattern variability this would need to be confirmed for each sample in the future.

In eight of the samples, 4-OH-CB187 was the dominating congener. It ranged from 0.022 to 2.26 ng/g with a geometric mean of 0.202 ng/g for all samples. This was the main metabolite in plasma determined in previous studies (5, 191, 203) The main metabolite in the remaining sample was 4-OH-CB146. The geometric mean concentration for 4-OH-CB146 in all samples was 0.149 ng/g and ranged from 0.010 to 1.88 ng/g. The metabolite may be formed from CB146 via a direct oxygen insertion or from CB153 or CB138 via NIH shift of Cl in the hydroxylation step. It should be noted that if a metabolite is formed by a direct oxygen insertion, it would retain the precursor PCB number. If the metabolite is produced via a NIH shift of Cl, the metabolite PCB number will be different than that of the parent PCB.

Total quantitated OH-PCBs ranged from 0.117 to 11.6 ng/g with a geometric mean value of 1.27 ng/g. Total OH-PCBs in individuals were equal to and up to 70 times that of the southern Quebec pooled sample (0.161 ng/g). Geometric mean CB153 and geometric mean total PCB concentrations in combined Inuit samples were 2.37 ng/g (range 0.263 to 13.9 ng/g) and 15.2 ng/g (range 1.19 to 65.9 ng/g), respectively. Concentrations in the southern Quebec pooled sample were 0.074 ng/g for CB153 and 0.488 ng/g for total PCBs. The mean ratio of total OH-PCBs to total PCBs was 0.11 (range 0.02 to 0.45) for combined Inuit and 0.33 for the southern Quebec pooled sample, demonstrating the importance of OH-PCBs in the total PCB-related compounds in blood. The concentration of the five main OH-PCB metabolites

relative to total OH-PCBs and CB153 for both Inuit and the southern Quebec pooled sample are shown graphically in Figure 6.3.

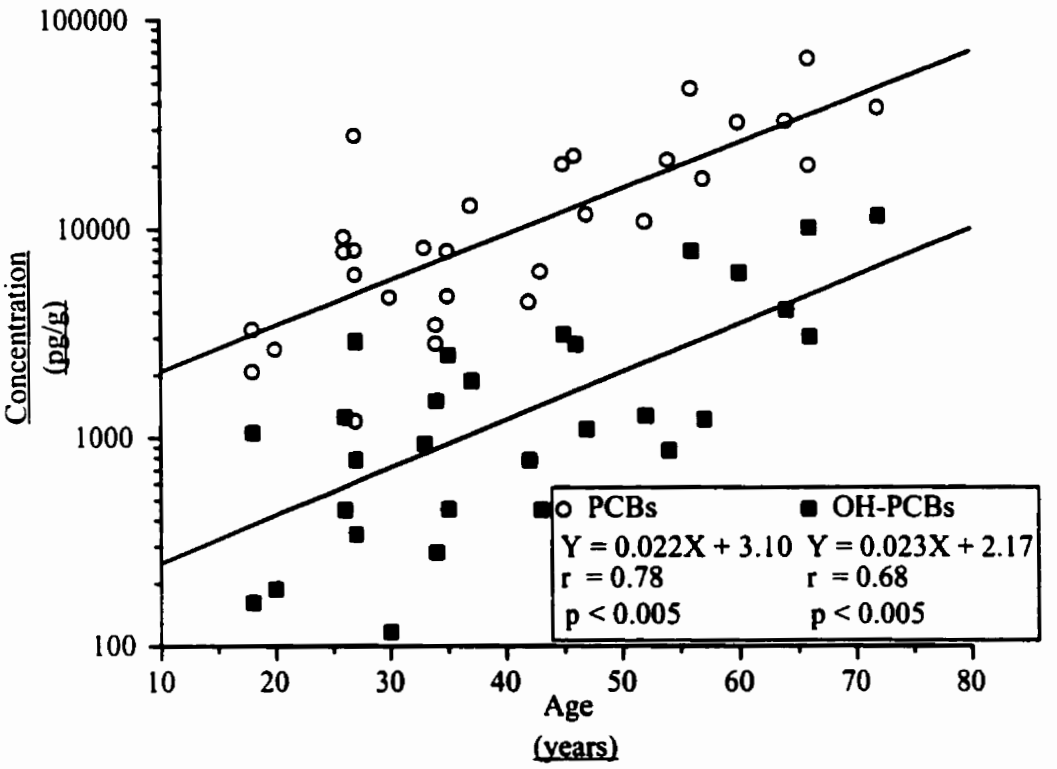


Figure 6.4 - Log transformed concentrations versus age (years) for total PCBs and total OH-PCBs.

Plots of log transformed concentrations versus age are shown in Figure 6.4. Both PCBs ($r = 0.78$) and OH-PCBs ($r = 0.68$) were found to be significantly ($p<0.005$) correlated with age. As seen by the similar slopes, total OH-PCBs and total PCBs were also significantly correlated ($r = 0.84$, $p < 0.005$).

6.4. Discussion

The only human blood analyzed previously for OH-PCBs and other chlorinated phenolic compounds was plasma from the Swedish population (5, 201).

PCP was described as being the dominant compound in these studies but levels were not given. The geometric mean PCP concentration in the Inuit samples was 2.02 ng/g, approximately three times lower than in the southern Quebec pooled sample. Geyer *et al.* (301) measured PCP in two different general populations in Germany and found average concentrations to be approximately 20 ng/g wet weight. The study did not indicate if the samples analyzed were whole blood or plasma so comparison of levels with the present results may be incorrect. However, a recent study has documented levels of PCP in men from Sweden and Latvia between 170 and 1800 ng/g on a plasma lipid weight basis (192). These levels are the same order of magnitude and have a similar range as those for the Inuit and the southern Quebec pooled whole blood samples when the Swedish data is approximated to plasma equivalents. This was estimated by assuming that whole blood is approximately half plasma by weight and that the average plasma lipid levels in the population are ca. 1%.

Although the concentration of PCP is lower in Inuit than that measured in the southern Quebec pooled whole blood sample, it may still play a role in the disruption of thyroid hormone transport. PCP binds to TTR with twice the affinity of the native hormone (72) and is responsible for 14-89% of the total phenolic compounds quantitated. Geyer *et al* (301) calculated the half-life of PCP to be approximately 19 days and concluded that the German cohort's calculated intake of PCP exceeded the elimination rate and approximated a bioconcentration factor of 1.4 for blood. This is likely due to binding with TTR and explains the high levels of PCP found in blood. Considering the significance of PCP in the chlorinated phenolic fraction, further investigation of PCP and its possible effects on thyroid hormone transport is required,

especially for southern populations, where levels of PCP far exceed OH-PCBs and other chlorinated phenolic compounds.

Another major compound in the Inuit whole blood chromatogram is 4-OH-HpCS, a previously unidentified chlorinated phenolic compound. At the time of analysis, an authentic standard was not available for quantitation. The major ion cluster for 4-OH-HpCS, using the given mass spectrometry conditions, was an isotope pattern indicative of a hexachlorinated compound. Therefore, the levels were approximated using the Cl_6 response factor from the OH-PCB standard mixture. Based on approximate concentrations, 4-OH-HpCS ranged from 1 to 17% of the total chlorinated phenolic compounds in the Inuit. Levels occasionally exceeded those of the main OH-PCBs.

The chromatogram of the phenolic compound fraction consists of more than 30 peaks that were identified as hydroxylated metabolites of PCBs based on their mass spectra. These represent on average 20% of the total chlorinated phenolic compounds in Inuit whole blood (range 2-56%). OH-PCBs constitute a significantly more important proportion of the total chlorinated phenolic compounds in Inuit compared to the general population pooled sample, in which OH-PCBs represent only 1.2% of the estimated total.

The mean concentration of the five main OH-PCBs (1.47 ng/g) in Inuit samples is slightly higher than that of a Swedish general population cohort (1.0 ng/g) (201). The Swedish data have been adjusted to whole blood wet weight equivalents. It was expected that levels of OH-PCBs in Inuit would be higher than found, since the mean PCB levels in plasma were 3 times that of this particular Swedish population

and an average of 30 times (range 2.5-133) that of the southern Quebec pooled sample. The range of the sum of these 5 congeners in the Swedish study (0.35 to 1.65 ng/g) is small compared to concentrations in the Inuit population, which range from 0.059 to 7.07 ng/g plasma wet weight levels of OH-PCBs. The higher levels of OH-PCBs are likely due to the higher levels of parent PCBs. OH-PCBs were found to be significantly correlated with PCBs ($r = 0.84$, $p < 0.005$), as expected.

The strong linear relationship observed between concentrations of PCBs and their metabolites suggests that no changes in enzyme activity affecting rate of formation or saturation of OH-PCBs binding to plasma proteins is taking place for this range of PCB exposure. The concentration of total quantitated chlorinated phenolic compounds was less than 2% of the theoretical molar circulating concentration of TTR in an average human (118). Thus, it is not likely that the concentration of total chlorinated phenolic compounds reached the carrying capacity of the blood, assuming that Inuit have similar TTR concentrations to the published values from other populations (118).

Women had lower mean concentrations of all phenolic compounds quantitated than men. The generally lower levels in women may result from the loss of OH-PCBs and PCBs through lactation, since both have been identified in milk (209, 302, 303). Both OH-PCBs and PCBs were significantly ($r = 0.68$ and 0.78, $p < 0.005$) correlated with age. The increase of both PCB and OH-PCB concentrations with age may be due to the increased exposure with age (elders' preference for traditional foods), a slow excretion rate of PCBs which prevents steady state from being achieved, or both. A

positive association between age and PCB levels in breast milk was reported previously in southern Quebec women (304) and Inuit adults from Nunavik (299).

The concentration of omega-3 fatty acids in plasma phospholipids has been shown to be a good indicator of fish intake (305). In the course of the *Santé Quebec Health Survey*, polyunsaturated fatty acid analysis was performed on plasma samples obtained from 492 Inuit adults. There was a strong association between omega-3 fatty acid content in plasma phospholipids and the age of the participants, with mean (geometric) concentrations of 6.4% (95%-CI: 5.9-6.9), 8.7% (8.2-9.3) and 12.2% (11.5-13.0) for the 18-24 years, 25-44 years and 45-74 years age groups, respectively (Fisher test: $p < 0.0001$) (*41*). Thus, increase in intake of traditional foods with age is indicated, and is probably partly responsible for the increase in PCBs with age. However, indications of increasing concentration of PCBs with age in other populations suggest that slow excretion of PCBs is also important. .

The main OH-PCBs identified in previous studies were also the dominating congeners in the Inuit population (see Figure 6.2). The major metabolite in 21 of the 30 samples analyzed was 4-OH-CB109. This was not the major metabolite found in the study by Bergman *et al.* (5) but it was later identified by Klasson-Wehler *et al.* (201) to be the dominant OH-PCB in another study of human plasma. The probable mechanism of formation of this metabolite is direct oxygen insertion into CB-109 or an NIH shift of Cl through an arene oxide intermediate of CB-118 or CB105. CB118 and CB105 are major congeners of Aroclor mixtures while CB-109 represents only a fraction of a percent of the Aroclor mixture, therefore the NIH shift mechanism is more likely and is known to occur in mink and mouse (*42*). The majority of

metabolites were highly correlated to all PCB congeners, making it impossible to determine most probable precursors by correlation analysis. For example, 4-OH-CB109 was significantly correlated to both CB105 and CB118 ($r = 0.67$ and 0.72, $p < 0.005$) The correlation coefficients for 4-OH-CB109 and unrelated PCBs, such as CB153 and CB187, were equally high (0.77 and 0.71, $p < 0.005$). This was true for the majority of the identified metabolites and the major PCBs in the Inuit whole blood samples.

The main metabolite in most of the remaining samples was 4-OH-CB187, which was previously identified as the major metabolite in a Swedish population (5). This compound was also identified as the main metabolite for the southern Quebec pooled sample and in other species including polar bear (203) and albatross (191). This metabolite is most likely formed from the metabolism of CB183 and/or CB187 (6), which represent 2 and 5% of the Aroclor 1254 mixture (*43*).

The OH-PCB pattern present in the chlorinated phenolic compound fraction in plasma is complex and it is difficult to generalize patterns in humans from such a small data set. As can be seen in Figure 6.3, the ratio of 4-OH-CB109 to total OH-PCBs ranged from 0.12 to 0.62 (mean of 0.34). The remaining congeners in the Inuit all composed similar proportions of total OH-PCBs compared to the southern population. The ratio of the 5 main congeners to CB153 in the southern pooled samples was within the range of those determined for the Inuit samples, except for 4-OH-CB187, where the southern pooled sample was higher than all the Inuit samples.

A number of factors, which include exposure to different proportions of precursor PCBs, alteration of metabolism rates by induction of hepatic enzymes,

inhibition of the metabolizing enzymes and the protein binding specificity of the plasma may all influence the retention of hydroxylated metabolites. Genetic diversity among individuals and populations may also influence the metabolism rates and binding specificity.

To evaluate the toxicological significance of the phenolic fraction, the main compounds must be identified, accurately quantitated and toxicological studies undertaken to evaluate the effects of these compounds on thyroid hormone and retinol homeostasis and binding at receptor sites. PCP remains the dominating phenolic compound in Inuit whole blood, even more so for the southern Quebec pooled sample. Concentrations of PCP in most of the samples far exceeded the main OH-PCBs. Other compounds, such as the unknown chlorinated compound, will be researched further to elucidate structure and possible roles in disruption of thyroid hormone transport.

In conclusion, total OH-PCB concentrations were 11% and 33% of total PCB concentrations in Inuit and a southern Quebec pooled sample of whole blood, respectively. Both total PCB and total OH-PCB geometric mean concentrations were higher for Inuit than the southern Quebec pooled sample and the current literature values (201). Increased concentrations resulted in increased OH-PCB levels in Inuit blood but the ratio of metabolites to PCBs was not as high as that found for the southern Quebec pooled sample. Unlike PCBs, the pattern of OH-PCBs was not consistent among individuals. Three different congeners alternated as the dominant metabolite of PCBs in the thirty samples analyzed. A larger study would be necessary to help elucidate the reasons for the pattern variability, the lower ratio of OH-PCBs to PCBs in Inuit, and the possible differences between the genders. PCP was found to be

the dominant chlorinated phenolic compound in the majority of the samples and must be included in future studies that may be evaluating the effects of phenolic compounds on circulating levels of thyroid hormones and vitamin A.

6.5. Acknowledgements

The author acknowledges Environment Canada and the National Wildlife Research Center for their support of this research. The project was funded by the Canadian Chlorine Coordinating Committee (C4) and the Canadian Chemical Producers Association.

Chapter 7. PCP and hydroxylated metabolites of PCBs in umbilical cord plasma from coastal populations of Quebec

7.1. Background

When it was determined that PCBs could effectively pass through the placental barrier and that they were associated with lower birth weights (306), research on the possible deleterious effects of PCBs on newborns and infants increased dramatically. Jacobson *et al.* (307) found that intrauterine exposed children had delayed central nervous system functioning. It was confirmed that, for this same cohort, reductions in cognitive function were associated with higher *in utero* PCB exposure at 4 years of age (308), followed by lower IQs at 11 years of age (309). All this evidence indicates a link between PCBs and neurodevelopment. Although many theories exist on how PCBs affect neurodevelopment, the main hypothesis involves PCB impact on thyroid hormone homeostasis (310). Thyroid hormones regulate neuronal proliferation, cell migration and differentiation including control on when differentiation begins and when cell proliferation ends (311). The main transport mechanism of thyroid hormones to the brain requires passing through the blood brain barrier via a thyroid hormone transport protein called transthyretin (TTR) (123). Although PCBs show some binding affinity for TTR (144), hydroxylated metabolites of PCBs (OH-PCBs) have much higher *in vitro* binding affinities that can be as high as 12x the binding affinity of the natural ligand, thyroxine (T4) (105, 142, 146). Binding to TTR is not limited to OH-PCBs. Other chlorinated phenolic compounds such as pentachlorophenol (PCP), halogenated phenols and brominated flame retardants (72,

145, 148) also have strong affinities for TTR. Recently, PCP was found to be the dominant phenolic compound, determined in Inuit whole blood (151). Other halogenated phenolic compounds may also be important contaminants in plasma (201) as they have been found to exhibit similar toxicological properties to OH-PCBs (72, 152, 190).

OH-PCBs have been shown to decrease circulating levels of thyroid hormones through this mode of thyroid hormone disruption (102, 157, 166). TTR is also responsible for retinol transport by forming a dimer with retinol binding protein. Thus, circulating retinol concentrations can also be affected by PCB and OH-PCB exposure (101, 106, 140).

The fetus may be especially vulnerable to PCB and OH-PCB exposure. When fetal mice were exposed *in utero* to 4′-OH-CB-79, a metabolite of CB77, both maternal and fetal plasma levels decreased significantly compared to controls (171). In this same study, fetal plasma had two times the 4′-OH-CB79 concentration of the maternal plasma (55). These experiments were recently repeated on pregnant rats who were orally exposed to 4-OH-CB109 (173), one of the main OH-PCBs found in human plasma (151, 192). In this study, both maternal and fetal thyroid hormones were reduced by exposure to 4-OH-CB109, with fetal total T4 concentrations decreasing to 89% of that of the controls (173). The decreased plasma T4 levels also resulted in decreased forebrain and cerebellum T4 concentrations as compared to controls (173), which may lead to a neurodevelopmental effect. PCP has also been shown to decrease brain T4 availability in dosed rats (174). Another interesting

finding for the 4-OH-CB109 rat dosing study was an accumulation of 4-OH-CB109 in fetal plasma, liver and brain.

Thus, prenatal exposure to PCBs, OH-PCBs, and PCP may all lead to thyroid hormone disruption and possibly neurodevelopmental effects. Umbilical cord plasma is unique in that it is a direct indication of *in utero* circulating concentrations of xenobiotics, including phenolic compounds, in the newborn infant. PCBs have been previously measured in umbilical cord plasma (282, 312-314), however, few studies have examined levels of hydroxylated metabolites in blood, especially in humans. OH-PCBs have been quantitated recently in Inuit whole blood (151) and Swedish and Latvian fish eaters (192). This study is the first to examine chlorinated phenolic compounds in umbilical cord plasma and to examine possible differences among three human populations with different PCB exposures due to cultural differences in dietary habits. Retinol and thyroid hormone status (triiodothyronine (T3), free T4, thyroid stimulating hormone (TSH), and thyroxine binding globulin (TBG)) were determined in most samples so the relationship between chlorinated phenolic compounds and these biological measures could be explored.

7.2. Materials & Methods

Plasma samples were obtained during various umbilical cord blood surveys conducted from 1993 to 1996 in Quebec (315, 316). These surveys took place in Nunavik (Northern Quebec), the Lower North Shore of the Gulf of St. Lawrence, and southern Quebec (Quebec City). The latter site represents a population with PCB exposure characteristic of the general population of Canada, while the former two sites

represent mostly aboriginal people whose diets may include a large quantity and variety of wildlife species. Nunavik diets may include marine mammal muscle and blubber, which have a higher concentration of PCBs than herbivores, including wildlife such as caribou, store-bought meats and dairy products (317, 318). The lower north shore group are subsistence fishermen, and may also eat seabird eggs (318). Ten samples from each region were randomly selected for chlorinated phenolic compound and PCB residue analysis from all samples collected during the survey. Nunavik samples were all from Inuit newborns, southern Quebec samples from Caucasians newborns and the Lower North Shore samples from three Caucasians and seven First Nations people.

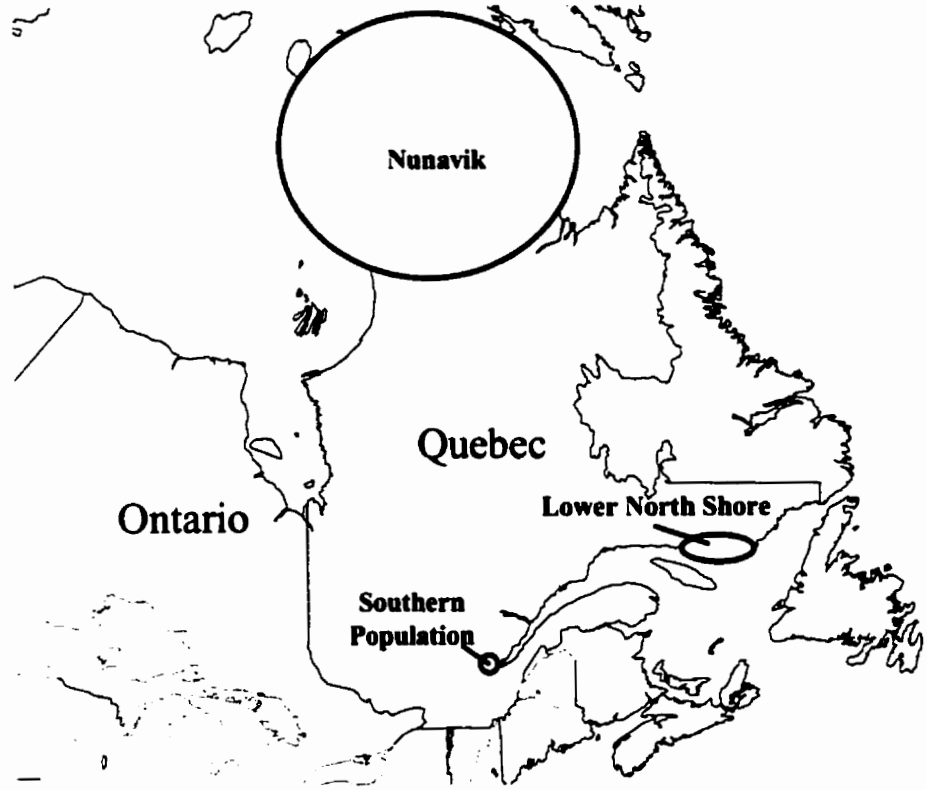


Figure 7.1 - Sampling locations for the umbilical cord plasma samples.

PCBs are numbered according to the corrected IUPAC numbering scheme as described by Ballschmiter and Zell (1) with corrections by Guitart *et al.* (2). Hydroxylated PCBs and their methoxylated derivatives are given the appropriate IUPAC PCB number according to their chlorination pattern. The OH- or MeO- functional groups are numbered thereafter.

The following $^{13}C_{12}$ labeled standards were acquired from Wellington Laboratories (Guelph, Ontario, Canada) and were used as an internal recovery standard mixture: 4′-OH-CB120, 4′-OH-CB159, 4′-OH-CB172 and 4-OH-CB187. PCP ($^{13}C_6$) was acquired from Cambridge Isotope Laboratories (Andover, MA) and was used for PCP quantitation. Labeled PCBs ($^{13}C_{12}$ - CB-118, 153, 180 and 194) were used as internal recovery standards and $^{13}C_{12}$ CB-138 as the performance standard for PCB analysis. $^{13}C_{12}$ PCB standards were purchased from Cambridge Isotope Laboratories (Andover, MA). The OH-PCB performance standard, 4′-Me-4-MeO-2,3,3′,5,6-pentachlorobiphenyl was a custom synthesis by Bob Wightman (Carleton University, Ottawa, ON).

A thorough description of the methodology and instrumentation used for these analyses was described in Chapter 2, Chapter 3 and Chapter 6. Methodology was altered slightly for this study. Umbilical cord plasma samples ranged from 1.63 to 10.44 grams and samples were spiked with 20 µl $^{13}C_{12}$ OH-PCB internal standard mixture (100 pg/µl), 20 µl $^{13}C_6$ PCP (100 pg/µl) and with 10 µl $^{13}C_{12}$ PCB internal standard mixture (2.5 ng/µl) prior to extraction. The final volume for the phenolic compound fraction was 25 µl and was spiked with 4′-Me-4-MeO-2,3,3′,5,6-

pentachlorobiphenyl as performance standard prior to analysis. The PCB fraction was brought down to a final volume of 100 µl and spiked with 10 µl CB138 ($^{13}C_{12}$) performance standard prior to analysis. Due to low levels of PCBs in the umbilical cord plasma samples, PCBs were analyzed by GC-MS(ECNI) using the same mass spectrometry conditions as described previously for OH-PCBs (151). Only pentachlorinated PCB congeners and higher are reported, since tetrachlorinated congeners and lower do not respond well to this type of detection. The gas chromatography temperature program remained the same as described previously (151). Congener specific analysis using a characterized Aroclor 1:1:1 quantitation mixture allowed the quantitation of 49 PCB congeners in most of the samples.

All solvents were residue analysis grade and purchased from EM Science (Gibbstown, NJ, USA). Merck Silica gel (Grade 60, 70-230 mesh, 60A) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). H_2SO_4 (Trace metal grade) was purchased from Fisher Scientific (Pittsburgh, PA).

Retinol analysis was performed at the *Quebec Toxicology Centre*. Ethanol was added to the plasma sample to denature proteins and retinol was extracted from the resulting solution with hexane. The hexane extract was concentrated under vacuum (Speed-Vac) and re-dissolved in ethanol. Retinol was determined by inverse phase high-pressure liquid chromatography (Waters Corp., Milford, Massachusetts) using a C-18 column and a UV detector (325 nm). Free T4, total T3, TSH and TBG were measured by heterogeneous competitive magnetic separation assay (Bayer Immuno 1^{TM} System), while TBG was determined by radioimmunoassay (IRMA PASTEUR). Thyroid hormones and TBG determinations were conducted at the *Unité de Recherche*

en Génétique Humaine (CHUL -CHUQ, Sainte-Foy, Quebec). Thyroid hormone measures were performed on all Nunavik and Lower North Shore samples and on only one southern Quebec sample.

All statistical analysis was completed with STATISTICA for Windows - version 5.1 from StatSoft, Inc. (1997) (Tulsa, OK). For statistical analysis, half the detection limit was used for compounds that were not detected in the samples.

7.3. Results

Recoveries of the internal recovery standards ($^{13}C_6$ PCP, and $^{13}C_{12}$ OH-PCBs and PCBs) were in the range of 75% – 104%. Mean recovery of all phenolic compound internal standards was greater than 87%. All concentrations were recovery corrected.

Using Liliefors test for normal distribution, the chemical residue data was not normally distributed. Thus, all data (including retinol and thyroid hormone concentrations) was log transformed prior to statistical analysis. The regional concentration data is summarized using geometric means along with minimum and maximum values.

Thirty compounds were characterized as OH-PCBs in the umbilical cord plasma samples. PCP and identified OH-PCB congeners are listed by region (n=10 for each) in Table 7.1. Two congeners, 4-OH-CB109 and 4′-OH-CB107 co-elute and were quantitated as a single peak. The peak is predominantly 4-OH-CB109 as demonstrated in other studies (5). Total OH-PCBs represent a sum of concentrations of all identified OH-PCBs and all compounds characterized as OH-PCBs.

Unidentified OH-PCBs were quantitated using relative response factors as described previously (151). Total OH-PCBs were analyzed for regional differences by MANOVA analysis. The Lower North Shore samples had the highest mean concentration of OH-PCBs and were significantly higher than the southern Quebec samples using the Sheffe test ($p = 0.01$). The Nunavik samples were not significantly different from the southern samples ($p = 0.8$) or the Lower North Shore samples ($p = 0.06$). PCP concentrations were similar and were not significantly different among the regions.

Forty-nine PCB congeners with five or more chlorines were above detection in the majority of the umbilical cord plasma samples. Total PCBs and CB153 are listed by region in Table 7.1. Table 7.1 also includes retinol, thyroid hormone and TBG concentrations and the ratio of total OH-PCBs to total PCBs. All quantitated PCB congeners are listed in Table 7.2. Total PCBs represent the sum of concentrations of all forty-nine congeners.

Table 7.1 - PCP, OH-PCBs, CB153 and total PCB geometric mean concentrations for the three regions (pg/g wet weight plasma, n=10 each region). Retinol and thyroid hormone and transport protein concentrations for Nunavik and Lower North Shore samples and one Southern Quebec sample are also shown. Italized OH-PCBs are tentative identifications based on the review by Letcher *et al.* (230). Note that 4-OH-CB109 and 4'-OH-CB107 co-eluted and they were quantitated as a single peak.

	Nunavik			Lower North Shore			Southern Population		
	Geometric Mean	Range min.	max.	Geometric Mean	Range min.	max.	Geometric Mean	Range min.	max.
(pg/g wet weight)									
PCP	1870	889	7680	1430	628	3640	1740	1020	4090
OH-PCBs									
4'-OH-CB120	2	1	4	7	3	20	2	0	6
4-OH-CB109 / 4'-OH-CB107	12	3	44	49	6	168	11	3	43
3-OH-CB153	19	4	65	23	10	74	6	3	14
4-OH-CB146	37	4	134	81	16	507	12	4	58
3'-OH-CB138	10	3	35	22	9	92	5	3	16
4'-OH-CB130	1	0	3	2	0	27	1	0	3
3-OH-CB187	4	0	34	7	2	21	1	0	3
4-OH-CB187	47	13	155	95	54	250	28	10	97
3'-OH-CB180	2	0	14	5	1	23	1	0	3
4'-OH-CB172	10	3	43	20	8	75	4	1	11
4-OH-CB193	3	1	17	3	1	8	1	0	5
4'-OH-CB199	0	0	4	0	0	7	0	0	0
4,4'-diOH-CB202	6	3	15	5	1	13	4	3	17
4'-OH-CB208	2	1	5	3	1	11	1	0	3
Total OH-PCBs	286	103	788	553	238	1750	234	147	464
CB153	262	49	1336	430	107	1350	104	83	199
Total PCBs	1510	309	6230	2710	525	7720	843	290	1650
OH-PCBs:PCBs	0.19	0.08	0.41	0.20	0.08	0.56	0.19	0.04	0.46
Retinol (nmol/l)	555	213	859	558	311	1020	661	366	1170
FT4 (pmol/l)	16.3	12.6	22.2	16.6	9.6	21.2	17.6	-	-
T3 (nmol/l)	0.64	0.45	1.23	0.49	0.20	0.78	0.70	-	-
TSH (µmol/l)	7.71	3.94	19.5	6.66	3.88	15.3	9.53	-	-
TBG (nmol/l)	924	592	1340	879	623	1350	640	-	-

Table 7.2 - Umbilical cord PCB concentrations (pg/g plasma wet weight) of all 49 quantitated congeners as categorized by region (n=10 each region) (n.d. = non-detect, < 1 are below detection limits for quantitation).

	Nunavik			Lower North Shore			Southern Population		
	Geometric Mean	Range min.	max.	Geometric Mean	Range min.	max.	Geometric Mean	Range min.	max.
CB92	9	n.d.	60	7	n.d.	167	9	n.d.	13
CB84	27	5	141	36	6	251	16	n.d.	31
CB101/90	49	11	262	87	13	786	44	17	181
CB99	100	16	1120	174	17	1630	38	n.d.	116
CB97	10	n.d.	167	12	n.d.	232	8	n.d.	19
CB87	28	5	236	63	8	436	13	n.d.	27
CB85	6	2	115	14	2	150	6	n.d.	86
CB110	42	8	403	79	10	709	44	18	502
CB118	67	19	402	155	30	673	35	9	81
CB105	19	6	300	37	7	155	11	2	31
CB136	13	1	316	15	2	651	12	1	536
CB151	7	3	34	11	4	68	8	n.d.	14
CB144/135	17	n.d.	97	27	n.d.	713	13	n.d.	22
CB149	20	9	71	33	n.d.	96	30	14	103
CB134	8	1	35	11	2	86	3	n.d.	18
CB146	23	5	98	54	15	178	11	6	54
CB153	262	49	1340	430	107	1350	104	30	199
CB141	3	2	20	5	3	13	5	1	8
CB130	6	2	20	9	3	30	2	n.d.	4
CB137	4	1	13	6	2	15	2	n.d.	3
CB138/163	157	36	712	232	62	704	54	11	110
CB158	5	2	18	8	2	17	3	1	6
CB178	1	n.d.	9	1	n.d.	3	1	n.d.	1
CB128	7	3	27	16	6	47	4	n.d.	7
CB156	27	5	94	40	17	104	11	2	19
CB157	8	2	28	17	7	45	5	n.d.	8
CB179	2	1	5	2	n.d.	4	2	n.d.	5
CB176	1	n.d.	2	1	n.d.	1	1	n.d.	2
CB178	2	< 1	27	1	< 1	20	1	n.d.	4
CB187/182	39	7	146	102	24	297	38	13	226
CB183	14	4	135	23	6	57	7	2	12
CB185	< 1	< 1	2	1	n.d.	1	1	n.d.	2
CB174	3	2	12	4	2	9	6	1	11
CB177	5	2	13	10	4	18	4	1	7
CB171	3	1	7	6	2	13	2	1	4
CB172	2	< 1	10	6	2	14	1	n.d.	3
CB180	118	33	663	146	43	501	40	8	84
CB193	3	< 1	53	5	1	23	1	< 1	4
CB191	< 1	< 1	5	1	< 1	7	1	< 1	2
CB170/190	21	4	74	39	13	87	13	3	22
CB202	4	1	21	5	3	10	2	n.d.	3
CB200	1	< 1	22	2	1	4	1	n.d.	2
CB199	1	n.d.	4	1	n.d.	4	2	n.d.	6
CB201	4	1	17	10	2	31	7	4	11
CB196/203	8	2	26	33	7	113	15	6	95
CB195	3	1	28	5	1	32	2	n.d.	2
CB194	9	2	23	17	7	55	11	2	21
CB206	3	1	10	6	3	12	1	n.d.	2
CB209	1	< 1	1	1	< 1	2	< 1	n.d.	1
Total PCBs	1510	309	6230	2703	525	7720	843	290	1650

Total PCBs were highest in Lower North Shore plasma samples and Nunavik samples but only Lower North Shore samples were significantly different ($p = 0.01$) from southern samples by the Sheffe test. The mean ratio of total OH-PCB metabolites to PCBs was highest in southern Quebec samples and lowest in Nunavik samples but the ratio was not statistically different among regions. Mean retinol concentrations were lowest in Nunavik samples and highest in southern samples but differences between the regions were not statistically significant. None of the thyroid hormone measures were significantly different between the Lower North Shore and the Nunavik samples.

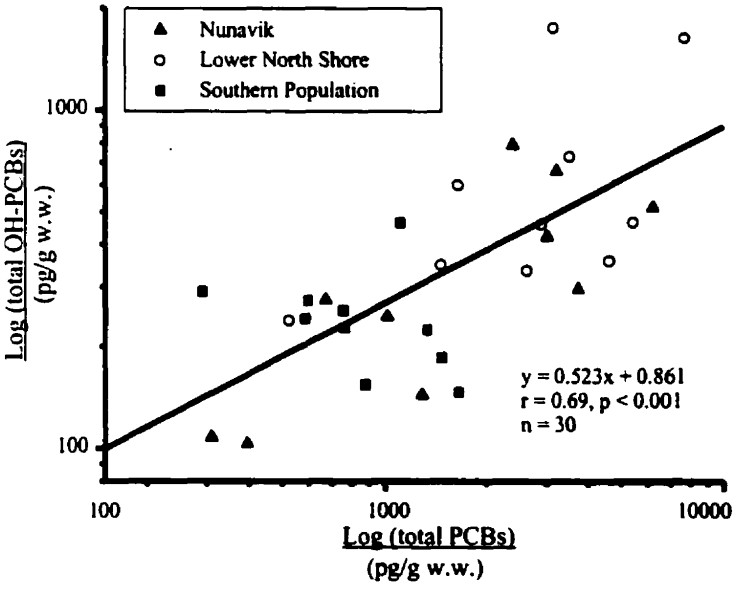


Figure 7.2 - Relationship between log-transformed total OH-PCBs and total PCBs concentrations for all regions.

The major OH-PCBs are formed by the hydroxylation of relatively persistent PCBs (5). Therefore, it is not surprising that log ΣOH-PCBs and log ΣPCBs are related in umbilical cord plasma, as shown in Figure 7.2 ($r = 0.69$, $p < 0.001$). The

relationship between non-transformed concentrations is non-linear. Concentrations of ΣOH-PCBs increase approximately as the square root of ΣPCBs concentrations which may indicate that the threshold of enyme induction has been reached and production of OH-PCBs from PCBs is no longer linear.

Individual OH-PCBs can be formed from multiple parent PCB congeners. Ratios of the main identified OH-PCBs relative to total OH-PCBs are given in Figure 7.3. The precursor PCBs that form the main metabolites are also given in Figure 7.3 and are shown as ratios to total PCBs. This figure demonstrates the relative proportion of the most abundant OH-PCBs analyzed to their potential parent compounds.

Figure 7.4 shows the correlation between two of the main OH-PCBs (4-OH-CB109 and 4-OH-CB146) and their potential precursor PCBs. Both log transformed metabolite concentrations were significantly correlated ($p < 0.001$) with all possible log transformed concentrations of precursor PCBs and were significantly correlated with many non-related PCBs (not shown).

Retinol was measured in all the umbilical cord samples analyzed in this study. No significant correlations were observed between retinol and any of the individual OH-PCB or PCB congeners nor to total OH-PCBs, total chlorinated phenolic compounds, or total PCBs.

Thyroid hormone measures, such as T3, free T4, TSH and TBG were measured for all Nunavik and Lower North Shore samples and only one sample from the southern Quebec samples (i.e. n=21). The main OH-PCB and PCB congeners and total OH-PCBs and PCBs were not significantly correlated with any of the thyroid hormone measures.

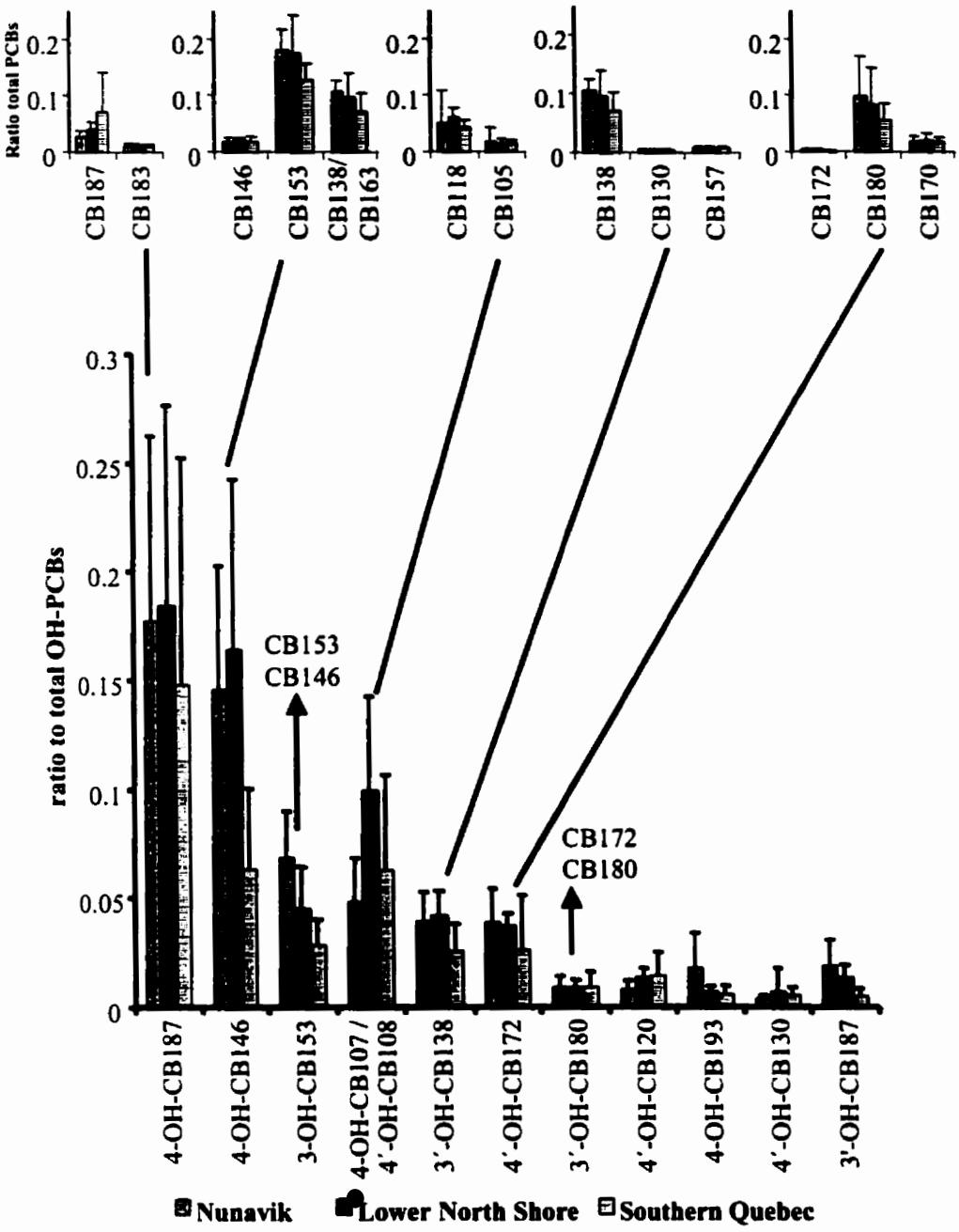


Figure 7.3 - The main identified OH-PCBs in umbilical cord plasma expressed as the mean percentage of total OH-PCBs. The possible precursor PCBs determined in umbilical cord plasma are also shown above the metabolites that they form and are expressed as the mean percentage of total PCBs. Error bars in the figure represent the standard deviation from the mean.

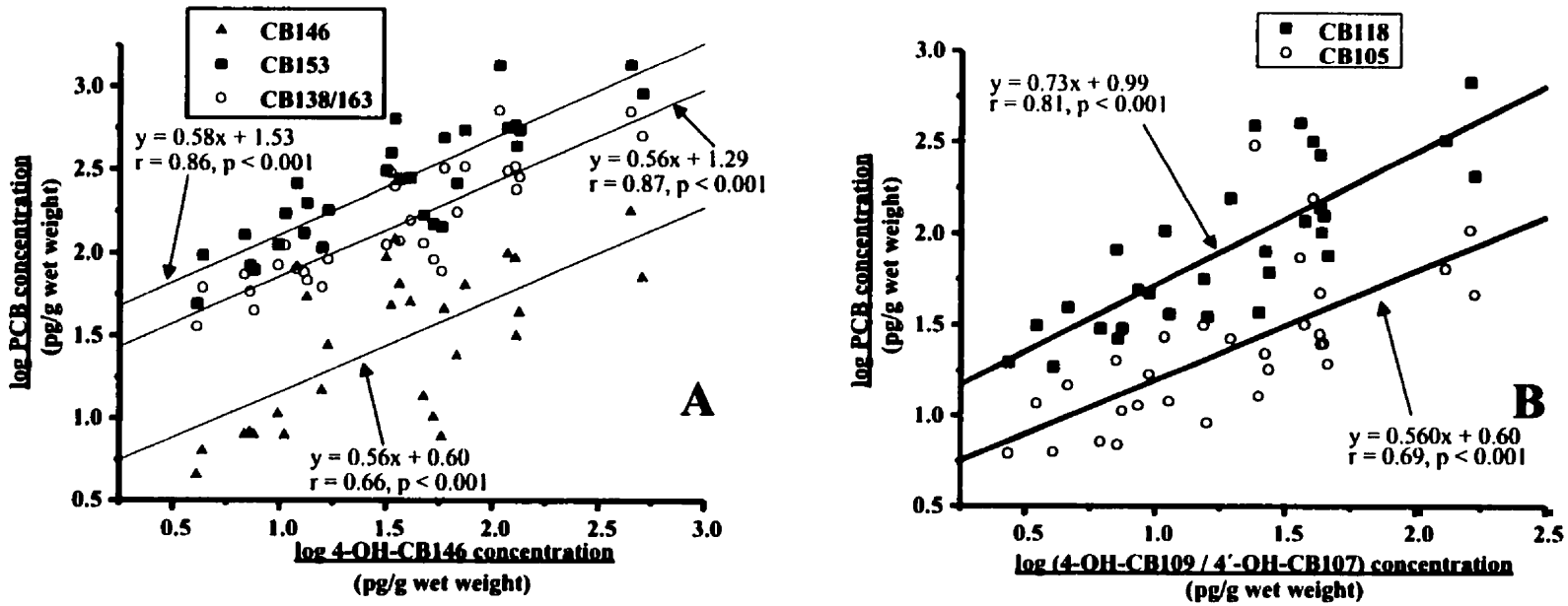


Figure 7.4 - Log normalized plots of potential precursor PCBs versus metabolites in umbilical cord plasma. A - Note that CB138 co-elutes with CB163 and they were quantitated as a single peak. B - Note that 4-OH-CB109 co-elutes with 4'-OH-CB107 and they were quantitated as a single peak.

PCP was negatively associated with T3 (r=-0.55, p=0.01), TBG (r=-0.44, p=0.05) and free T4 (r=-0.51, p=0.02). Assuming that all chlorinated phenolic compounds display similar effects, Figure 7.5 demonstrates the statistically significant inverse association (r = -0.62, p = 0.003) between log transformed free T4 and log transformed total chlorinated phenolic compounds (sum PCP and OH-PCBs). Log transformed total chlorinated phenolic compounds were also negatively associated with log transformed total T3 (r = -0.48, p = 0.03).

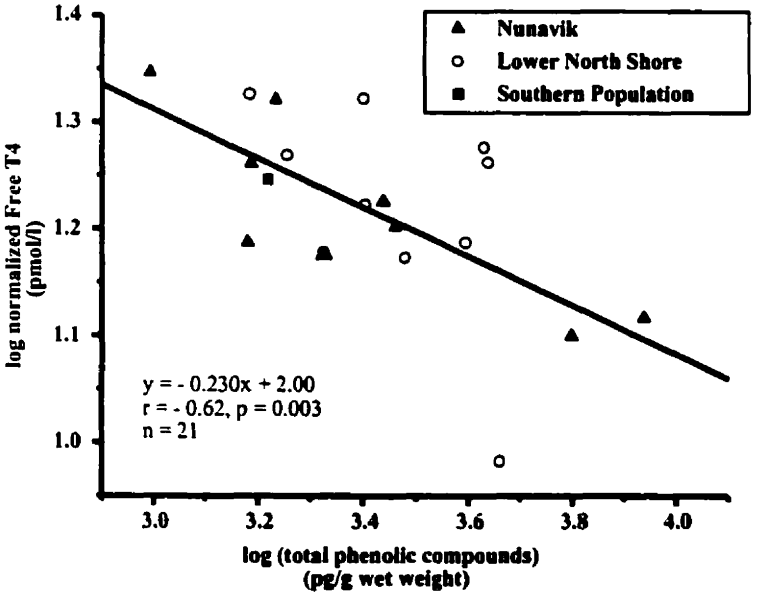


Figure 7.5 - The significant negative association between log transformed free T4 concentrations and log transformed concentration of the total chlorinated phenolic compounds (sum PCP and total OH-PCBs).

7.4. Discussion

This is the first study to examine hydroxylated metabolites and other chlorinated phenolic compounds in umbilical cord plasma. PCP was the most abundant phenolic compound in all samples analyzed, regardless of region. All three

regions had similar concentrations. Mean PCP concentrations were not significantly different by MANOVA analysis (Table 7.1). The concentration of PCP ranged from 628 to 7680 pg/g wet weight in all the samples analyzed. The ratio of PCP to CB153 for all samples ranged from 0.72 to 42.3 indicating the relative significance of PCP among the samples. PCP constituted an average of 78%, 66% and 82% of the total quantitated chlorinated phenolic compounds in the Nunavik, Lower North Shore and southern Quebec samples, respectively. Thus, PCP is the dominant chlorinated phenolic contaminant in umbilical cord plasma in all of the populations and may supercede OH-PCBs as the chlorinated phenolic compound of highest concern in humans. Plasma is the most important compartment for PCP storage. In dosed rats, it was shown that 99% of PCP in blood is tightly bound to plasma proteins (71). In human test subjects, the percentage of PCP bound to plasma proteins was estimated to be 96% (319).

PCP and its salts have been used extensively as wood preservatives, biocides and disinfectants (320). PCP use has been curtailed since the late 1970s and even banned in some countries, such as Sweden (1977) and Germany (1987) (320). In Canada, PCP is regulated by the Pest Control Products Act and its use has been restricted since 1981, in order to minimize human exposure through food, cutaneous contact and inhalation. The main exposure to PCP for non-occupationally exposed individuals is through diet (321). Another significant source of PCP may also occur through the metabolism of hexachlorobenzene (70).

Because PCP does not bioaccumulate in fatty tissues and is rapidly excreted in dosed animals (71), it is often overlooked and rarely determined in routine

organochlorine analysis. We previously reported PCP as the dominant halogenated phenolic compound in blood samples from Nunavik and southern Quebec adults (151) and therefore PCP should be added to the list of compounds analyzed in future human monitoring studies.

PCP can induce deleterious effects on several organs or tissues. Increased lymphocyte responses were noted in patients with high PCP blood levels (322). PCP can be metabolized to reactive quinone metabolites (323) with possible covalent binding to crude liver homogenates and isolated liver proteins *in vitro* (324). PCP has twice the affinity of T4 to TTR (72) and has been shown to decrease circulating T4 levels in rams exposed from conception (325). PCP has also been shown to affect thyroid hormone metabolism by competitively inhibiting iodothyronine sulfation *in vitro* (326). In the present study, plasma concentration of total phenolic compounds, the major part being PCP, was negatively correlated to free T4 and total T3 plasma levels. A 9 fold increase in total phenolic compounds resulted in 2 fold and 6 fold decreases in free T4 and T3 plasma levels, respectively. This suggests that PCP and perhaps other chlorinated phenolic compounds can alter thyroid hormone status in newborns, which in turn could lead to adverse neurodevelopmental effects in infants.

Another compound recently identified as a major chlorinated phenolic compound in polar bear plasma, 4-hydroxy-heptachlorostyrene (4-OH-HpCS) (147), was also found in the human umbilical cord plasma samples. This compound was determined in all umbilical cord plasma samples. No quantitative standard was available at the time of analysis but concentrations of 4-OH-HpCS were estimated using the average heptachloro-MeO-PCB response factor from the quantitated

methoxylated PCB standards. The geometric mean concentrations for Nunavik, Lower North Shore and southern Quebec samples were 29, 34 and 5 pg/g wet weight plasma, respectively. This is the first time this compound has been shown to be present in human plasma. The likely precursor for this compound is octachlorostyrene (OCS), which is an industrial by-product. The low concentrations of 4-OH-HpCS in the general population compared to the higher concentrations in Nunavik and Lower North Shore samples indicates that OCS exposure is mainly a result of a diet including wild animals as opposed to an average Canadian diet. Sandau *et al.* (147) showed that this compound had a binding affinity to TTR similar to that of T4, which is slightly less than PCP (72) and lower than those determined for OH-PCBs (58).

Concentrations of total OH-PCBs in umbilical plasma were highest in the Lower North Shore samples as shown in Table 7.1. Over 30 compounds were identified as OH-PCBs, of which 11 were positively identified with authentic standards. Three more have been identified by Bergman *et al.* (231) but are only tentatively identified in these samples until authentic standards can be obtained to confirm their identities.

Figure 7.2 demonstrates the relationship between log total OH-PCBs and log total PCBs. The slope of the relationship (m = 0.52) is lower than the slope determined for Inuit whole blood (m = 1.72) in Chapter 6 (151). It is not clear why the relation between OH-PCBs and PCBs is not as strong for umbilical cord plasma as for Inuit whole blood. It is interesting to note that the ratio of total OH-PCBs to total PCBs was similar among the regions, ca. 0.2. The ratio in this study was twice that found in a previous study involving the whole blood of Canadian Inuit (0.11) (151). It

has been shown previously that PCBs are most concentrated in the lipoproteins of plasma (243). The higher ratio of OH-PCBs to PCBs in umbilical cord plasma samples than whole blood may reflect the difference in constitution of adult and umbilical cord plasma. For example, umbilical cord plasma has approximately half the lipid content and less transthyretin than adult plasma (327). The lower transthyretin concentrations in umbilical cord plasma compared to adult plasma may explain the non-linear relationship between the OH-PCB and PCB concentrations, as evidenced by a slope significantly less than 1 in Figure 7.2. PCP concentrations may be high enough to saturate the available TTR causing a decreased carrying capacity of the plasma for OH-PCBs. TTR concentrations have been found to be approximately 1.1 mg/ml in umbilical cord plasma (327). TTR has a molecular weight of 55 kDa, which gives a TTR concentration of 20 nmol/ml for umbilical cord plasma. Concentration of total phenolic compounds ranged from 3-30 pmol/ml (assuming 1 g plasma = 1 ml plasma). Thus, it is not likely that total quantitated phenolic compounds are saturating the umbilical cord TTR carrier proteins.

Another possible explanation for the differences could involve placental transfer of OH-PCBs. The passage of PCB congeners through the placenta has been shown to be congener specific and to increase with the degree of chlorination (328). This is likely due to the different chemical properties of each of the congeners and the lower lipid content of umbilical cord plasma compared to maternal plasma (327). It is unclear whether OH-PCBs are affected in a similar fashion since OH-PCBs have never been determined in both maternal and umbilical cord plasma. The transfer of PCB metabolites from dosed mice to the fetus was tested by Sinjari *et al.* (55). They

showed that 4'-OH-CB79 concentrations in fetal plasma were twice that of the maternal plasma, 24 hours post-exposure, which indicates enhanced transport of metabolite, likely through binding to TTR.

The main metabolite in 27 of the 30 samples was 4-OH-CB187. This congener has previously been determined as the dominant metabolite in fish eaters from Sweden, Black footed and Laysan Albatross and polar bear (191, 192, 203). Two possible parent PCBs (shown in Figure 7.3) can form 4-OH-CB187 through two different hydroxylation mechanisms. The first involves the direct insertion (329) of oxygen to form a hydroxyl group at the *para* position of CB187. Direct insertion has been demonstrated to occur for *in vitro* metabolism studies of halobenzenes (330, 331) and CB52 (50) as well as in an *in vivo* study involving CB187 dosed rats (6, 332, 333). CB187 is an abundant congener found in biota and is found as a small percentage (0.54%) in the Aroclor 1254 mixture (9). It is more abundant in Aroclor 1260, representing ca. 5.4% of the total (9). The second mechanism of oxidation is the formation of a 3,4 (meta-para) - epoxide in CB183 followed by a 3,4 shift of chlorine to the meta position similar to the NIH shift first described by Guroff *et al.* (47). Epoxide formation in the metabolism of PCBs has been demonstrated in *in vitro* studies (48) as well as *in vivo* studies (102) using CB77 as substrate. CB183 constitutes approximately 0.2% and 2.4% of Aroclor 1254 and 1260 mixtures, respectively (9). Both CB187 and CB183 were quantitated in the umbilical cord plasma samples. They compose between 1% and 14% of the total PCBs detected in the plasma samples for all regions (Figure 7.3). The plasma concentration of the main metabolite is a function of its rate of formation from PCB precursors and its retention

in blood due to specific binding to TTR. Specific binding is likely the main reason for the relatively high concentrations determined, as metabolic formation is thought to be a very slow process. This slow process does not effect the toxicokinetics of the compound as much as clearance via partitioning into fecal matter, which is quantitatively the most rapid process of PCB clearance of slowly metabolized PCBs in homeotherms (334).

The second most abundant metabolite in umbilical cord plasma was 4-OH-CB146. This metabolite can be formed by direct insertion onto CB146 or by the NIH shift of chlorine during metabolism of CB138 or CB153. These three parent PCBs comprise between 20 and 30% of the total PCBs quantitated in all the samples. CB153 and CB138 are the two most abundant PCBs determined in the plasma samples (Figure 7.3) and are major components in Aroclor mixtures (9). The three likely parent PCBs were significantly ($p < 0.001$) correlated with 4-OH-CB146 (Figure 7.4). The correlation coefficients for CB138, CB153 and CB146 were 0.86, 0.87 and 0.66, respectively. The correlation was not as strong for CB146 due to some samples being at or very close to detection limits.

The third most abundant metabolite was 4-OH-CB109, which can be formed from CB107 (direct insertion), CB105 (NIH-Cl shift) or CB118 (NIH-Cl shift). Both CB105 and CB118 are major congeners in Aroclor 1254, composing 5.2% and 10.5% of the total (9). CB107 is a minor congener in Aroclor 1254 (0.6%) and it is rarely determined in environmental samples, including these umbilical cord plasma samples. The parent PCBs (CB105 and CB118) were also significantly correlated ($p < 0.001$) with their possible metabolite (4-OH-CB109) with correlation coefficients of 0.69 and

0.81 (Figure 7.4). The 4-OH-CB109 was previously found to be the main metabolite in adult Inuit whole blood, Latvian fish consumers, Baltic seals, white tailed eagle and rats dosed with Aroclor 1254 (5, 151, 192, 231).

The relationship between metabolites and their precursor PCBs could not be further resolved by using multiple step regression analysis (forward or backward). All major metabolites were highly intercorrelated with all PCBs, even unrelated PCBs.

As seen in Figure 7.3, the top three metabolites in the Lower North Shore samples constituted a higher percentage of the total OH-PCBs as compared to the other sampling sites. Hydroxylated PCB patterns have been shown to vary among individuals (151, 192). The variation between individuals is a function of both retention and formation of metabolites. The retention of specific OH-PCBs is probably similar for all humans. The main structural requirement for retention is the capability to bind to TTR (142). This requirement is thought to involve a hydroxyl group with adjacent chlorines (58). Generally, the hydroxyl group is in the para position of the biphenyl ring, but not exclusively since meta substituted metabolites are also found in plasma. TTR is a highly conserved transport protein that is present in most species (335). Humans have varying concentrations of TTR in plasma and some genetic abnormalities are known (118) but generally fall within a certain range and are in excess molar concentration to OH-PCBs (151). Thus, the main deciding factor for the pattern of OH-PCBs in blood is the formation of metabolites from the pattern of parent PCB exposure.

When the chemical residue data was compared to thyroid hormone and vitamin A measures, only PCP was significantly associated with total T3, free T4, and TBG.

PCP has been shown to affect thyroid hormone concentrations in sheep (325, 336, 337) and rats (338). Assuming that all phenolic compounds exert similar affects on thyroid hormone homeostasis, when all phenolic compounds were summed and compared to the thyroid hormone measures, only total T3 (not shown) and free T4 (Figure 7.5) were negatively associated. Reduced plasma total T3 and T4 concentrations may be a result of induced UDP-GT enzymes in the liver causing increased metabolism of the thyroid hormones (130). The negative association between free T4 and total phenolic compounds is also in agreement with the theory of OH-PCBs and other halogenated phenolic compounds binding to TTR and disrupting thyroid hormone transport. The sample sizes of this experiment are too small to draw significant conclusions but these results support previously determined hypotheses of thyroid hormone disruption by PCBs and/or their metabolites. The fact that PCP is associated with free T4 and that the addition of other phenolic compounds increased the significance of the association demonstrates that chlorinated phenolic compounds may be acting through a common mechanism. Many common mechanisms have been determined for both OH-PCBs and PCP. Both OH-PCBs and PCP bind with high affinity to human TTR in *in vitro* studies (72, 142) and inhibit thyroid hormone metabolism (152, 153, 326).

Morse *et al.* (162) found that both maternal and neonatal rats showed decreased total and free T4 levels with exposure to CB169 and/or CB77 in a dose dependent manner. They concluded that fetal T4 levels were affected by both a reduction in transplacental delivery of T4 and increased T4 metabolism by the induced glucuronyltransferase enzymes. Darnerud *et al.* (172) also demonstrated fetal

reduction in total T4 and free T4 when pregnant mice were dosed with CB77. In a human exposure study (312), Dutch infants showed decreased free and total T4 levels with increased PCB/dioxin toxic equivalency factors. Thus, many studies indicate that T4 concentrations can be decreased with exposure to PCBs and this study supports the theory that PCP and OH-PCBs may be partly responsible for this decrease.

TTR has been shown to be an important mechanism of T4 transport in cerebral spinal fluid to the brain (123). If chlorinated phenolic compounds can significantly alter plasma T4 levels by disrupting transport via TTR binding, this may lead to brain thyroid hormone deficiencies *in utero* possibly affecting brain development (339). TTR is also important in thyroid hormone transport across the placental barrier (340). Maternal sources of thyroid hormones are thought to influence fetal brain development (341). The binding of metabolites to TTR may also be a significant mode of transport for phenolic compounds to penetrate the placental barrier, as shown in mice (171). Thus, phenolic compounds may be able to disrupt maternal sources of thyroid hormones, penetrate into fetal circulation and disrupt local thyroid hormone supply in the developing fetus. The potential of PCP and OH-PCBs to disrupt thyroid hormone homeostasis for the developing fetus warrants further investigation to confirm the observed effects of this study. A study that is currently underway includes a larger cohort and will examine the relationship between phenolic compounds and thyroid hormone and retinol concentrations in newborns.

7.5. Acknowledgements

The project was funded by the Canadian Chlorine Coordinating Committee (C4) and the Canadian Chemical Producers Association. The author acknowledges Environment Canada and the National Wildlife Research Center for their support of this research. All biochemical data was supplied by Pierre Ayotte and Eric Dewailly (Centre de Recherche du CHUL- Centre Hospitalier Universitaire de Quebec, Beauport, PQ, Canada). I would like Jason Duffe for his help with some of the statistical analysis of the data.

Chapter 8. Summary and Future Research

The main ideas and conclusions from this thesis can be summarized by the following points:

1. New extraction and quantitation technique for the OH-PCBs found in plasma and whole blood

An efficient method of extraction of phenolic compounds was developed and applied to human, polar bear and ringed seal plasma samples. The liquid:liquid extraction method is long and solvent intensive but adequate for the extraction of both neutral and phenolic compounds from plasma. Using GC-ECNI-MS allowed unequivocal identification of known compounds and the determination of unknown compounds using relative response factors. The use of SIM-MS allowed peak determination of closely eluting peaks using different monitoring ions that would not be possible using conventional ECD detection. The new method of analysis allowed the quantitation of 13 congeners in most of the samples analyzed. No other studies in the literature have reported the accurate quantitation of this many congeners.

2. Identification of a new contaminant that may implicate the importance of monitoring background level compounds

In the survey of OH-PCBs in polar bear plasma, a metabolite of octachlorostyrene was discovered. The metabolite was found to have the same properties as other halogenated phenolic compounds by binding to TTR with 1.1 times the affinity of the natural hormone, T4. This further supports the theory that OH-PCBs and other

halogenated phenolic compounds are selectively retained through binding to TTR. The discovery of 4-OH-HpCS also stresses the importance of monitoring the presence of minor contaminants as their significance as potential endocrine-disrupting agents may be underestimated.

3. Pentachlorophenol is a major contributor to the phenolic compound fraction in human samples

PCP is a well-known contaminant that seems to have lost importance to the more persistent and bioaccumulating compounds such as PCBs, DDT and DDT metabolites. PCP is not a banned substance in North America and is still produced and used as a fungicide and pesticide. PCP was found to be the most important contaminants in human plasma samples. It possesses many toxicological properties such as binding to TTR (72), disruption of sulfotransferase activity for thyroid hormone metabolism (326) and inhibition estrogen metabolism (190). These effects coupled with the high concentrations found in human blood (Chapter 6) and umbilical cord plasma (Chapter 7) warrant increased monitoring of PCP in biota and human samples.

4. OH-PCBs are a major class of contaminants in a heavily exposed animal

Most species have plasma OH-PCB concentrations ranging from 5-30% of the total PCBs (151, 191, 192, 211). The polar bear, because of its contaminant load and its high metabolic capability, was able to produce OH-PCBs concentrations that were generally higher than PCB concentrations. These are the highest OH-PCB concentrations ever determined in plasma. With OH-PCBs at such high

concentrations, it may be important to further investigate OH-PCB effects on the organism level and possibly link the *in vitro* experiments to actually effects on polar bears themselves.

5. OH-PCBs can be associated with certain biological measures that are not associated with PCBs

The main hypothesis of the thesis was to relate some of the common biomarkers associated with PCB exposure to their hydroxylated metabolites. In umbilical cord plasma, total phenolic compounds were negatively associated with free T4 levels. This indicates that both PCP and OH-PCBs are utilizing a similar mode of action to reduce free T4 levels in newborns. In polar bears, OH-PCBs were found to be positively associated with plasma retinol concentrations while being negatively associated with the free T4 index. The mechanism for positive association with retinol is unknown. Negative association with the free T4 index is in agreement with current theories of OH-PCBs occupying potential thyroid hormone binding sites and may explain the higher concentration of OH-PCBs found in Svalbard polar bears. Further research is needed to confirm these results and explore the possibility of gender and age differences in these polar bear relationships.

Future Research

Accurate quantitation of OH-PCBs requires identification and quantitation of all metabolites found in plasma. We were only able to identify 13 congeners of the

estimated 35 congeners found in polar bear plasma. More work is needed in the synthesis of OH-PCBs so that more congeners can be determined in the future.

In addition to OH-PCB identification, the identification of other halogenated phenolic compounds is also important. It was estimated that there are over 100 unknown halogenated phenolic compounds found in plasma (201) that all need to be identified and quantitated if the full potential of endocrine disruption by phenolic compounds is to be realized.

OH-PCBs may be selectively accumulated in plasma but are not restricted to the plasma compartment. Recently, OH-PCBs have been determined in adipose and liver (210). This study found that the pattern of OH-PCBs in adipose and liver was quite different than those found in plasma. Determination of OH-PCBs in other matrices such as adipose, liver and milk may help evaluate the mechanisms of OH-PCB toxicity by better understanding their toxicokinetics. It will also help determine the threat of lactational transfer to developing infants.

OH-PCBs may not be limited to the biotic environment. OH-PCBs may also be found in the abiotic environment. Little is known about the physical chemical properties of OH-PCBs. It is assumed that OH-PCBs are easily excreted from an organism and subsequently lost to the environment. OH-PCBs are likely stable molecules and may be found in soil, sediment, and water. Further research into the ecodynamics of OH-PCBs is needed to help evaluate these properties and should start with the determination of the physicochemical properties of OH-PCBs.

Lastly, many toxicological effects have been demonstrated for OH-PCBs in *in vivo* experiments. OH-PCBs seem to be highly active having both estrogenic (190)

and thyroidogenic properties (136). Continued testing is needed to evaluate other possible modes of action such as steroidogenic activity or effects on mitochondrial respiration.

Bibliography

1. Ballschmiter K, Zell M. 1980. Analysis of polychlorinated biphenyls (PCB) by glass capillary gas chromatography. Composition of technical Aroclor- and Clophen-PCB mixtures. *Fresenius' Zeitschrift für Analytische Chemie* 302:20-31

2. Guitart R, Puig P, Gómez-Catalán J. 1993. Requirement for a standardized nomenclature criterium for PCBs: Computer-assisted assignment of correct congener denomination and numbering. *Chemosphere* 27(8):1451-1459

3. Hebert CE, Hobson KA, Shutt JL. 2000. Changes in food web structure affect rate of PCB decline in herring gull (*Larus argentatus*) eggs. *Environmental Science & Technology* 34(9):1609-1614

4. McKinney JD. 1998. Do residue levels of polychlorinated biphenyls (PCBs) in human blood produce mild hypothyroidism? *Journal of Theoritical Biology* 129:231-241

5. Bergman Å, Klasson-Wehler E, Kuroki H. 1994. Selective retention of hydroxylated PCB metabolites in blood. *Environmental Health Perspectives* 102(5):464-469

6. Sjödin A, Tullsten AK, Klasson-Wehler E. 1998. Identification of the parent compounds to selectively retained hydroxylated PCB metabolites in rat blood plasma. *Organohalogen Compounds* 37:365-368 (Extended Abstract)

7. Hutzinger O, Safe S, Zitko V. 1974. *The Chemistry of PCB's*. Cleveland: CRC Press, Inc. pp.1-269

8. Frame GM. 1999. Improved procedure for single DB-XLB column GC-MS-SIM quantitation of PCB congener distributions and characterization of two different preparations sold as "Aroclor 1254". *Journal of High Resolution Chromatography* 22(10):533-540

9. Frame GM, Cochran JW, Bøwadt SS. 1996. Complete PCB congener distributions for 17 Aroclor mixtures determined by 3 HRGC systems optimized for comprehensive, quantitative, congener specific analysis. *Journal of High Resolution Chromatography* 19:657-668

10. Jensen S. 1966. Report of a new chemical hazard. *New Scientist* 32:612

11. Jensen S, Johnels AG, Olsson M, Otterlind G. 1969. DDT and PCB in marine animals from Swedish waters. *Nature* 224(216):247-250

12. Wania F, Mackay D. 1993. Global fractionation and cold condensation of low volatility organochlorine compounds in polar regions. *Ambio* 22:10-18

13. Ballschmiter K, Zell M. 1980. Baseline studies of the global pollution. I. Occurrence of organohalogens in pristine European and antarctic aquatic environments. *International Journal of Environmental Analytical Chemistry* 8(1):15-35

14. Elder DL, Fowler SW. 1977. Polychlorinated biphenyls: penetration into the deep ocean by zooplankton fecal pellet transport. *Science* 197(4302):459-461

15. Jan J, Malnersic S, Faganeli J. 1978. Chlorinated hydrocarbons (pesticides and polychlorinated biphenyls-- PCBs) in the atmosphere of Ljubljana. *Arhiv za Higijenu Rada I Toksikologiju* 29(1):15-20

16. Moore SA, Jr., Harriss RC. 1972. Effects of polychlorinated biphenyl on marine phytoplankton communities. *Nature* 240(5380):356-358

17. Clausen J, Braestrup L, Berg O. 1974. The content of polychlorinated hydrocarbons in Arctic mammals. *Bulletin of Environmental Contamination & Toxicology* 12(5):529-534

18. McFarland VA, Clarke JU. 1989. Environmental occurrence, abundance, and potential toxicity of polychlorinated biphenyl congeners: Considerations for a congener- specific analysis. *Environmental Health Perspectives* 81:225-239

19. Jensen S, Sundstrom G. 1974. Stuctures and levels of most chlorobiphenyls in two technical PCB products and in human adipose tissue. *Ambio* 3:70-76

20. Guengerich FP. 1993. Cytochrome P450 enzymes. *American Scientist* 81:440-447

21. Ortiz de Montellano, PR. 1995. *Cytochrome P450 - Structure, Mechanism and Biochemistry*. New York: Plenum Press. pp.1-652

22. Walker CH. 1980. Species variations in some hepatic microsomal enzymes that metabolize xenobiotics. In *Progress in drug metabolism*, ed. Bridges JW, Chasseaud LF, John Wiley & Sons Ltd. 2: pp.112-164

23. Borlakoglu JT, Walker CH. 1989. Comparative aspects of congener specific PCB metabolism. *European Journal of Drug Metabolism and Pharmacokinetics* 14(2):127-131

24. Boon JP, Van Arnhem E, Jansen S, Kannan N, Petrick G, Schulz D, Duinker JC, Reijnders PJH, Goksoyr A. 1992. The toxicokinetics of PCB's in marine mammals with special reference to possible interactions of individual congeners with the cytochrome P450-dependent monooxygenase system: an overview. In *Persistent Pollutants in Marine Ecosystems*, ed. Walker CH, Livingstone DR, Oxford: Pergamon Press. 6: pp.119-160

25. Safe S. 1994. Polychlorinated biphenyl (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Critical Reviews in Toxicology* 24(2):87-149

26. Safe S, Robertson LW, Safe L, Parkinson A, Bandiera S, Sawyer T, Campbell MA. 1982. Halogenated biphenyls: molecular toxicology. *Canadian Journal of Physiology and Pharmacology* 60(7):1057-1064

27. Safe S. 1984. Polychlorinated biphenyls (PCBs) and polybrominated biphenyls (PBBs): biochemistry, toxicology, and mechanism of action. *Critical Reviews in Toxicology* 13(4):319-395

28. Safe S. 1989. Polyhalogenated aromatics: uptake, disposition, and metabolism. In *Halogenated biphenyls, terphenyls, naphthalenes, dibenzodioxins and related products*, ed. Kimbrough RD, Jensen AA, Amsterdam: Elsevier Science Publishers B.V. 5: pp.131-159

29. Denomme MA, Bandiera S, Lambert I, Copp L, Safe L, Safe S. 1983. Polychlorinated biphenyls as phenobarbitone-type inducers of microsomal enzymes. Structure-activity relationships for a series of 2,4-dichloro-substituted congeners. *Biochemical Pharmacology* 32(19):2955-2963

30. Honkakoski P, Negishi M. 1998. Regulatory DNA elements of phenobarbitol-responsive cytochrome P450 CYP2B genes. *Journal of Biochemical and Molecular Toxicology* 12(1):3-9

31. Savas U, Griffin KJ, Johnson EF. 1999. Molecular mechanisms of cytochrome P-450 induction by xenobiotics: An expanded role for nuclear hormone receptors. *Molecular Pharmacology* 56:851-857

32. Waxman DJ. 2000. P450 gene induction by structurally diverse xenochemicals: Central role of nuclear receptors CAR, PXR, and PPAR. *Archives of Biochemistry and Biophysics* 369:11-23

33. Waxman DJ, Azaroff L. 1992. Phenobarbital induction of cytochrome *P*-450 gene expression. *Biochemistry Journal* 281:577-592

34. Okey AB. 1990. Enzyme induction in the cytochrome P-450 system. *Pharmacology and Therapeutics* 4X:241-298

35. Denison MS, Whitlock JP, Jr. 1995. Xenobiotic-inducible transcription of cytochrome P450 genes. *The Journal of clinical endocrinology and metabolism* 270(31):18175-18178

36. Whitlock JP, Jr. 1995. Induction of cytochrome P450 enzymes that metabolize xenobiotics. In *Cytochrome P450 - Structure, mechanism, and biochemistry*, ed. Ortiz de Montellano PR, New York: Plenum Press. 10: pp.391-418

37. Hahn ME. 1998. The aryl hydrocarbon receptor: a comparative perspective. *Comparative Biochemistry and Physiology C: Comparative Pharmacology and Toxicology* 121(1-3):23-53

38. Bigelow SW, Nebert DW. 1982. The *Ah* regulatory gene product. Survey of nineteen polycyclic aromatic compounds' and fifteen benzo[a]pyrene metabolites' capacity to bind to the cytosolic receptor. *Toxicology Letters* 10:109-118

39. Landers JP, Bunce NJ. 1991. The *Ah* receptor and the mechanism of dioxin toxicity. *Biochemical Journal* 276(2):273-287

40. Okey AB, Riddick DS, Harper PA. 1994. Molecular biology of the aromatic hydrocarbon (dioxin) receptor. *Trends in Pharmacological Sciences* 15:226-232

41. Poland A, Bradfield C. 1993. The Ah locus: a review. In *Steroid Hormone Receptors*, ed. Moudgil VK, Boston: Birkhäuser. pp.503-509

42. Whitlock JPJr. 1993. Mechanistic aspects of dioxin action. *Chemical Research in Toxicology* 6:754-763

43. Boon JP, Eijgenraam F, Everaarts JM. 1989. A structure-activity relationship (SAR) approach towards metabolism of PCBs in marine animals from different trophic levels. *Marine Environmental Research* 27:159-176

44. Groves JT, Han Y-Z. 1995. Models and mechanisms of cytochrome P450 action. In *Cytochrome P450 - Structure, mechanism, and biochemistry*, ed. Ortiz de Montellano PR, New York: Plenum Press. 1: pp.3-48

45. Peterson JA, Graham-Lorence SE. 1995. Bacterial P450s: Structural similarities and functional differences. In *Cytochrome P450 - Structure, mechanism, and biochemistry*, ed. Ortiz de Montellano PR, New York: Plenum Press. 5: pp.151-180

46. Ortiz de Montellano PR. 1995. Oxygen activation and reactvity. In *Cytochrome P450 - Structure, mechanism, and biochemistry*, ed. Ortiz de Montellano PR, New York: Plenum Press. 8: pp.245-303

47. Guroff G, Daly JW, Jerina DM, Renson J, Witkop B, Udenfriend S. 1967. Hydroxylation-induced migration: the NIH shift. *Science* 157:1524-1530

48. Ishida C, Koga N, Hanioka N, Saeki HK, Yoshimura H. 1991. Metabolism *in vitro* of 3,4,3',4'-tetrachlorobiphenyl by rat liver microsomes and highly purified cytochrome P-450. *Journal of Pharmacobio-Dynamics* 14:276-284

49. Borlakoglu JT, Wilkins JPG. 1993. Metabolism of di-, tri- and tetrabromobiphenyls by hepatic microsomes isolated from control animals and animals treated with Aroclor 1254, a commercial mixture of polychlorinated biphenyls (PCBs). *Comparative Biochemistry and Physiology* 105C(1):107-112

50. Preston BD, Miller JA, Miller EC. 1983. Non-arene oxide aromatic ring hydroxylation of 2,2',5,5'- tetrachlorobiphenyl as the major metabolic pathway catalyzed by phenobarbitol-induced rat liver microsomes. *Journal of Biological Chemistry* 258(13):8304-8311

51. Sipes IG, Slocumb ML, Perry DF, Carter DE. 1982. 2,4,5,2',4',5'-Hexachlorobiphenyl: Distribution, metabolism, and excretion in the dog and monkey. *Toxicology and Applied Pharmacology* 65:264-272

52. Ariyoshi N, Koga N, Yoshimura H, Oguri K. 1997. Metabolism of 2,4,5,2',4',5'-hexachlorobiphenyl (PCB153) in guinea pig. *Xenobiotica* 27(9):973-983

53. Ariyoshi N, Koga N, Oguri K, Yoshimura H. 1992. Metabolism of 2,4,5,2',4',5'-hexachlorobiphenyl with liver microsomes of phenobarbital-treated dog: the possible formation of PCB 2,3-arene oxide intermediate. *Xenobiotica* 22(11):1275-1290

54. Ariyoshi N, Oguri K, Koga N, Yoshimura H, Funae Y. 1995. Metabolism of highly persistent PCB congener, 2,4,5,2',4',5'- hexachlorobiphenyl, by human CYP2B6. *Biochemical and Biophysical Research Communications* 212(2):455-460

55. Sinjari T, Klasson-Wehler E, Hovander L, Darnerud PO. 1998. Hydroxylated polychlorinated biphenyls: distribution in the pregnant mouse. *Xenobiotica* 28(1):31-40

56. Brouwer A, Ahlborg UG, van den Berg M, Birnbaum LS, Boersma ER, Bosveld B, Denison MS, Gray LE, Hagmar L, Holene E, Huisman M, Jacobson SW, Jacobson JL, Koopman-Esseboom C, Koppe JG, Kulig BM, Morse DC, Muckle G, Peterson RE, Sauer PJJ, Seegal RF, Smits-van Prooije AE, Touwen BCL, Weisglas-Kuperus N, Winneke G. 1995. Functional aspects of developmental toxicity of polyhalogenated aromatic hydrocarbons in

experimental animals and human infants. *European Journal of Pharmacology - Environmental Toxicology and Pharmacology Section* 293:1-40

57. Sundström G, Hutzinger O. 1976. The metabolism of 2,2',4,4',5,5'-hexachlororbiphenyl by rabbits, rats and mice. *Chemosphere* 4:249-253

58. Lans MC, Klasson-Wehler E, Willemsen M, Meussen E, Safe S, Brouwer A. 1993. Structure-dependent, competitive interaction of hydroxy-polychlorobiphenyls, -dibenzo-p-dioxins and -dibenzofurans with human transthyretin. *Chemico-Biological Interactions* 88(1):7-21

59. Sundström G, Hutzinger O. 1976. The metabolism of chlorobiphenyls - A review. *Chemosphere* 5:267-298

60. Garrison AW, Nzengung VA, Avants JK, Ellington JJ, Jones WJ, Rennels D, Wolfe NL. 2000. Phytodegradation of *p,p'*-DDT and the enantiomers of *o,p'*-DDT. *Environmental Science & Technology* 34(9):1663-1670

61. Risebrough RW, Rieche P, Peakall DB, Herman SG, Kirven MN. 1968. Polychlorinated biphenyls in the global ecosystem. *Nature* 220(172):1098-1102

62. Dearth MA, Hites RA. 1991. Complete analysis of technical chlordane using negative ionization mass spectrometry. *Environmental Science & Technology* 25:254-264

63. AMAP. 1998. Persistent Organic Pollutants. In *AMAP Assessment Report: Arctic Pollution Issues*,Oslo, Norway: Arctic Monitoring and Assessment Programme (AMAP). 6: pp.183-371

64. Muir DG, Norstrom RJ, Simon M. 1988. Organochlorine contaminants in Arctic marine food chains: accumulation of specific PCB congeners and chlordane-related compounds. *Environmental Science & Technology* 22(9):1071-1079

65. World Health Organization. 1984. *Chlordane - Environmental Health Criteria 34*. Geneva: World Health Organization. pp.3-82

66. Karlsson H. 1998. Isomer and enantiomer selective ultra trace analysis of chlordane in biota including isolation and structure elucidation of single congeners. Universität Basel. pp.1-153

67. Buser HR, Müller MD. 1993. Enantioselective determination of chlordane components, metabolites, and photoconversion products in environmental samples using chiral high-resolution gas chromatography and mass spectrometry. *Environmental Science & Technology* 1993(27):1211-1220

68. Li YF, Cai DJ, Singh A. 1998. Technical hexachlorocyclohexane use trends in China and their impact on the environment. *Arch. Environ. Contam Toxicol.* 35(4):688-697

69. Li YF, Barrie LA, Bidleman TF, McConnell LL. 1998. Global HCH use, trends and their impact on the arctic atmospheric environment. *Geophys. Res. Lett.* 25:39-41

70. Renner G. 1988. Hexachlorobenzene and its metabolism. *Toxicological and Environmental Chemistry* 18:51-78

71. Braun WH, Young JD, Blau GE, Gehring PJ. 1977. The pharmacokinetics and metabolism of pentachlorophenol in rats. *Toxicology and Applied Pharmacology* 41(2):395-406

72. van den Berg KJ. 1990. Interaction of chlorinated phenols with thyroxine binding sites of human transthyretin, albumin and thyroid binding globulin. *Chemico-Biological Interactions* 76:63-75

73. Pastukhov B, Kononov EYa, Halsall CJ, Bailey R, Stern GA, Barrie LA, Fellin P, Muir DCG, Rosenberg B, Rovinsky FYa. 1998. Multi-year observations of organohalogen pesticides in the Arctic atmosphere. *Environmental Pollution* 102(2):51-62

74. Morriss-Kay GM, Ward SJ. 1999. Retinoids and mammalian development. *International Review of Cytology* 188:73-131:73-131

75. Langer P, Tajtakova M, Fodor G, Kocan A, Bohov P, Michalek J, Kreze A. 1998. Increased thyroid volume and prevalence of thyroid disorders in an area heavily polluted by polychlorinated biphenyls. *European Journal of Endocrinology* 139(4):402-409

76. Goodman DS, Blaner WS. 1984. Biosynthesis, absorption, and hepactic metabolism of retinol. In *The Retinoids*, ed. Sporn MB, Roberts AB, Goodman DS, Orlando: Academic Press, Inc. 7: pp.2-41

77. Frolik CA. 1984. Metabolism of retinoids. In *The Retinoids*, ed. Sporn MB, Roberts AB, Goodman DS, Orlando: Academic Press, Inc. 11: pp.177-209.

78. Goodman DS. 1976. Retinol-binding protein, prealbumin, and vitamin A transport. *Progress in Clinical and Biological Research* 5:313-30:313-330

79. Goodman DS. 1985. Retinoids and retinoid-binding proteins. *The Harvey Lectures* 81:111-132

80. Blomhoff R, Green MH, Berg T, Norum KR. 1990. Transport and storage of Vitamin A. *Science* 250:399-404

81. Håkansson H, Hanberg A. 1989. The distribution of [^{14}C]-2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and its effect on the vitamin A content in parenchymal and stellate cells of rat liver. *Journal of Nutrition* 119:573-580

82. Zanotti G, Malpeli G, Berni R. 1993. The interaction of *N*-ethyl retinamide with plasma retinol-binding protein (RBP) and the crystal structure of the retinoid-RBP complex at 1.9-Å resolution. *Journal of Biological Chemistry* 268(33):24873-24879

83. Zanotti G, Marcello M, Malpeli G, Folli C, Sartori G, Berni R. 1994. Crystallographic studies on complexes between retinoids and plasma retinol-binding protein. *Journal of Biological Chemistry* 26(47):29613-29620

84. Cowan SW, Newcomer ME, Jones TA. 1990. Crystallographic refinement of human serum retinol binding protein at 2Å resolution. *Proteins: Structure, Function, and genetics* 8:44-61

85. Goodman DS. 1984. Plasma retionl-binding protein. In *The Retinoids*, ed. Sporn MB, Roberts AB, Goodman DS, Orlando: Academic Press, Inc. 8: pp.42-89

86. Goodman DS, Raz A. 1972. Extraction and recombination studies of the interaction of retinol with human plasma retinol-binding protein. *Journal of Lipid Research* 13:338-347

87. Malpeli G, Folli C, Berni R. 1996. Retinoid binding to retinol-binding protein and the interference with the interaction with transthyretin. *Biochimica et Biophysica Acta* 1294:48-54

88. Naylor HM, Newcomer ME. 1999. The structure of human retinol-binding protein (RBP) with its carrier protein transthyretin reveals an interaction with the carboxy terminus of RBP. *Biochemistry* 38(9):2647-2653

89. Monaco HL, Mancia F, Rizzi M, Coda A. 1994. Crystallization of the macromolecular complex transthyretin-retinol-binding protein. *Journal of Molecular Biology* 244:110-113

90. Walker CH. 1995. Biochemical biomarkers in ecotoxicology--some recent developments. *The Science of the Total Environment* 171(1-3):189-195

91. Zile MH. 1992. Vitamin A homeostasis endangered by environmental pollutants. *Proceedings of the Society for Experimental Biology and Medicine* 201(2):141-153

92. Grant DL, Moodie CA, Phillips WEJ. 1974. Toxicodynamics of Aroclor 1254 in the male rat. *Environmental Physiology & Biochemistry* 4(5):214-225

93. Innami S, Nakamura A, Miyazaki M, Nagayama S, Nishide E. 1976. Further studies on the reduction of vitamin A content in the livers of rats given polychlorinated biphenyls. *Journal of Nutritional Science and Vitaminology (Tokyo)* 22(6):409-418

94. Azais V, Pascal G, Arand M, Oesch F, Robertson LW. 1986. Effects of congeneric polychlorinated biphenyls on liver and kidney retinoid levels. *Chemosphere* 15(9-12):1905-1908

95. Jenssen BM. 1996. An overview of exposure to, and effects of, petroleum oil and organochlorine pollution in grey seals (Halichoerus grypus). *The Science of the Total Environment* 186(1-2):109-118

96. Azais V, Arand M, Rauch P, Schramm H, Bellenand P, Narbonne JF, Oesch F, Pascal G, Robertson L. 1987. A time-course investigation of vitamin A levels and drug metabolizing enzyme activities in rats following a single treatment with prototypic polychlorinated biphenyls and DDT. *Toxicology* 44:341-354

97. Zile MH, Bank PA, Roltsch IA. 1989. Alterations in vitamin A metabolism by polyhalogenated aromatic hydrocarbons. *Zeitschrift für Ernährungswissenschaft* 28(2):93-102

98. Brouwer A, van den Berg KJ. 1984. Early and differential decrease in natural retinoid levels in C57BL/Rij and DBA/2 mice by 3,4,3',4'-tetrachlorobiphenyl. *Toxicology and Applied Pharmacology* 73:204-209

99. Brouwer A, van den Berg KJ, Kukler A. 1985. Time and dose responses of the reduction in retinoid concentration in C57BL/Rij and DBA/2 mice induced by 3,4,3',4'-tetrachlorobiphenyl. *Toxicology and Applied Pharmacology* 78:180-189

100. Narbonne JF, Grolier P, Albrecht R, Azais V, Oesch F, Robertson LW. 1990. A time course investigation of vitamin A level and lipid composition of the liver endoplasmic reticulum in rats following treatment with congeneric polychlorobiphenyls. *Toxicology* 60:253-261

101. Brouwer A, Reijnders PJH, Koeman JH. 1989. Polychlorinated biphenyl (PCB)-contaminated fish induces vitamin A and thyroid hormone deficiency in the common seal (*Phoca vitulina*). *Aquatic Toxicology* 15:99-106

102. Brouwer A, van den Berg KJ. 1986. Binding of a metabolite of 3,4,3',4'-tetrachlorobiphenyl to transthyretin reduces serum vitamin A transport by inhibiting the formation of the protein complex carrying both retinol and thyroxin. *Toxicology and Applied Pharmacology* 85:301-312

103. Brouwer A, van den Berg KJ, Blaner WS, Goodman DS. 1986. Transthyretin (prealbumin) binding of PCBs, a model for the mechanism of interference with vitamin A and thyroid hormone metabolism. *Chemosphere* 15:1699-1706

104. Walker CH. 1998. The Use of Biomarkers to Measure the Interactive Effects of Chemicals. *Ecotoxicology and Environmental Safety* 40(1/2):65-70

105. Brouwer A. 1991. Role of biotransformation in PCB-induced alterations in vitamin A and thyroid hormone metabolism in laboratory and wildlife species. *Biochemical Society Transactions* 19(3):731-737

106. Brouwer A, Blaner WS, Kukler A, van den Berg KJ. 1988. Study on the mechanism of interference of 3,4,3',4'-tetrachlorobiphenyl with the plasma retinol-binding proteins in rodents. *Chemico-Biological Interactions* 68:203-217

107. Bartalena L, Robbins J. 1993. Thyroid hormone transport proteins. *Clinics in Laboratory Medicine* 13(3):583-598

108. Hoffenberg R, Ramsden DB. 1983. The transport of thyroid hormones. *Clinical Science* 65(4):337-342

109. Robbins J, Bartalena L. 1986. Plasma transport of thyroid hormones. In *Thyroid Hormone Metabolism*, ed. Hennemann G, New York: Marcel Dekker, Inc. 1: pp.3-38

110. Chopra IJ. 1991. Nature, sources, and relative biologic significance of circulating thyroid hormones. In *Werner and Ingbar's The Thyroid - A Fundamental and Clinical Text*, ed. Braverman LE, Utiger RD, Philadelphia: J.B. Lippincott Company. 7: pp.126-143

111. Stockigt JR. 1991. Serum thyrotropin and thyroid hormone measurements and assessment of thyroid hormone transport. In *Werner and Ingbar's - The thyroid: A fundamental and clinical text*, ed. Braverman LE, Utiger RD, Philadelphia: J.B. Lippincott Company. 19: pp.463-485

112. Kanda Y, Goodman DS, Canfield RE, Morgan FJ. 1974. The amino acid sequence of human plasma prealbumin. *The Journal of Biological Chemistry* 249(21):6796-6805

113. Blake CCF, Geisow MJ, Oatley SJ, Rérat B, Rérat C. 1978. Structure of prealbumin: Secondary, Tertiary and quaternary interactions determined by fourier refinement at 1.8 Å. *Journal of Molecular Biology* 121:339-356

114. Larsson M, Pettersson T, Carlström A. 1985. Thyroid hormone binding in serum of 15 vertebrate species: Isolation of thyroxine-binding globulin and prealbumin analogs. *General and Comparative Endocrinology* 58:360-375

115. Chang L, Munro SLA, Richardson SJ, Schreiber G. 1999. Evolution of thyroid hormone binding by transthyretins in birds and mammals. *European Journal of Biochemistry* 259:534-542

116. Schreiber G, Richardson SJ. 1997. The evolution of gene expression, structure and function of transthyretin. *Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology* 116(2):137-160

117. Makover A, Moriwaki H, Ramakrishnan R, Saraiva MJM, Blaner WS, Goodman DS. 1988. Plasma transthyretin - Tissue sites of degradation and turnover in the rat. *The Journal of Biological Chemistry* 263(18):8596-8603

118. Fielden MR, Chen I, Chittim B, Safe SH, Zacharewski TR. 1997. Examination of the estrogenicity of 2,4,6,2',6'-pentachlorobiphenyl (PCB 104), its hydroxylated metabolite 2,4,6,2',6'-pentachloro-4- biphenylol (HO-PCB 104), and a further chlorinated derivative, 2,4,6,2',4',6'-hexachlorobiphenyl (PCB 155). *Environmental Health Perspectives* 105(11):1238-1248

119. Eberhardt NL, Apriletti JW, Baxter JD. 1980. The molecular biology of thyroid hormone action. In *Biochemical Actions of Hormones*, New York: Academic Press, Inc. 9: pp.311-394

120. Robbins J. 1991. Thyroid hormone transport proteins and the physiology of hormone binding. In *Werner and Ingbar's The Thyroid - A Fundamental and Clinical Text*, ed. Braverman LE, Utiger RD, Philadelphia: J.B. Lippincott Company. 6: pp.111-125

121. Southwell BR, Tu GF, Duan W, Achen M, Harms PJ, Aldred AR, Richardson SJ, Thomas T, Pettersson TM, Schreiber G. 1992. Cerebral expression of transthyretin: evolution, ontogeny and function. *ACTA Medica Austrica* 19 Suppl 1:28-31:28-31

122. Schreiber G, Southwell BR, Richardson SJ. 1995. Hormone delivery systems to the brain-transthyretin. *Experimental and Clinical Endocrinology & Diabetes* 103(2):75-80

123. Chanoine JP, Braverman LE. 1992. The role of transthyretin in the transport of thyroid hormone to cerebrospinal fluid and brain. *ACTA Medica Austrica* 19 Suppl 1:25-8:25-28

124. Palha JA, Hays MT, Morreale dE, Episkopou V, Gottesman ME, Saraiva MJ. 1997. Transthyretin is not essential for thyroxine to reach the brain and other tissues in transthyretin-null mice. *American Journal of Physiology* 272(3 Pt 1):E485-E493

125. Nilsson C, Lindvall-Axelsson M, Owman C. 1992. Neuroendocrine regulatory mechanisms in the choroid plexus-cerebrospinal fluid system. *Brain Research. Brain Research Reviews* 17:109-138

126. Lee J, Ronald K, Øritsland NA. 1977. Some blood values of wild polar bears. *The Journal of Wildlife Management* 41(3):520-526

127. Visser TJ. 1994. Role of sulfation in thyroid hormone metabolism. *Chemico-Biological Interactions* 92:293-303

128. Miyashita K, Murakami M, Iriuchijima T, Takeuchi T, Mori M. 1995. Regulation of rat liver type 1 iodothyronine deiodinase mRNA levels by testosterone. *Molecular and Cellular Endocrinology* 115:161-167

129. Visser TJ. 1994. Sulfation and glucuronidation pathways of thyroid hormone metabolism. In *Thyroid hormone metabolism - Molecular biology and alternate pathways*, ed. Wu S-Y, Visser TJ, Boca Raton: CRC Press, Inc. 6: pp.85-117

130. Barter RA, Klaassen CD. 1992. UDP-glucuronosyltransferase inducers reduce thyroid hormone levels in rats by an extrathyroidal mechanism. *Toxicology and Applied Pharmacology* 113(1):36-42

131. Liu J, Liu Y, Barter RA, Klaassen CD. 1995. Alteration of thyroid homeostasis by UDP-glucuronosyltransferase inducers in rats: a dose-response study. *The Journal of Pharmacology and Experimental Therapeutics* 273(2):977-985

132. Hood A, Klaassen CD. 2000. Differential effects of microsomal enzyme inducers on *in vitro* thyroxine (T_4) and triiodothyronine (T_3) glucuronidation. *Toxicological Sciences* 55:78-84

133. Visser TJ, Kaptein E, Glatt H, Bartsch I, Hagen M, Coughtrie MW. 1998. Characterization of thyroid hormone sulfotransferases. *Chemico-Biological Interactions* 109(1-3):279-291

134. Chopra IJ, Santini F, Wu S-Y, Hurd RE. 1994. The role of sulfation and desulfation in thyroid hormone metabolism. In *Thyroid hormone metabolism - Molecular biology and alternate pathways*, ed. Wu S-Y, Visser TJ, Boca Raton: CRC Press, Inc. 7: pp.119-138.

135. Anderson RJ. 1994. Biochemical characterization of triiodothyronine sulfotransferase (T_3-ST). In *Thyroid hormone metabolism - Molecular biology and alternate pathways*, ed. Wu S-Y, Visser TJ, Boca Raton: CRC Press, Inc. 9: pp.155-174

136. Brouwer A, Morse DC, Lans MC, Schuur AG, Murk AJ, Klasson-Wehler E, Bergman Å, Visser TJ. 1998. Interactions of persistent environmental

organohalogens with the thyroid hormone system: mechanisms and possible consequences for animal and human health. *Toxicology and Environmental Health* 14(1-2):59-84

137. Brucker-Davis F. 1998. Effects of environmental synthetic chemicals on thyroid function. *Thyroid* 8(9):827-856

138. Marshall JS, Tompkins LS. 1968. Effect of o,p'-DDD and similar compounds on thyroxine binding globulin. *The Journal of clinical endocrinology and metabolism* 28(2):386-392

139. McKinney JD, Fawkes J, Jordan S, Chae K, Oatley S, Coleman RE, Briner W. 1985. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) as a potent and persistent thyroxine agonist: A mechanistic model for toxicity based on molecular reactivity. *Environmental Health Perspectives* 61:41-53

140. Brouwer A, Kukler A, van den Berg J. 1988. Alterations in retinoid concentrations in several extrahepatic organs of rats by 3,4,3',4'-tetrachlorobiphenyl. *Toxicology* 50:317-330

141. Lans MC. 1995. Thyroid hormones binding proteins as novel targets for hydroxylated polyhalogenated aromatic hydrocarbons (PHAHs): possible implications for toxicity. Doctorate Thesis thesis. Agricultural University, Wageningen, The Netherlands. pp.93-113-152

142. Lans MC, Spiertz C, Brouwer A, Koeman JH. 1994. Different competition of thyroxine binding to transthyretin and thyroxine-binding globulin by hydroxy-PCBs, PCDDs and PCDFs. *European Journal of Pharmacology - Environmental Toxicology and Pharmacology Section* 270(2-3):129-136

143. McKinney JD, Waller CL. 1994. Polychlorinated biphenyls as hormonally active structural analogues. *Environmental Health Perspectives* 102(3):290-297

144. Chauhan KR, Kodavanti PRS, McKinney JD. 2000. Assessing the role of *ortho*-substitution on polychlorinated biphenyl binding to transthyretin, a thyroxine transport protein. *Toxicology and Applied Pharmacology* 162:10-21

145. Meerts IATM, van Zanden JJ, Luijks EAC, van Leeuwen-Bol I, Marsh G, Jakobsson E, Bergman Å, Brouwer A. 2000. Potent competitive interactions of some brominated flame retardants and related compounds with human transthyretin *in vitro*. *Toxicological Sciences* 56(1):95-104

146. Cheek AO, Kow K, Chen J, McLachlan JA. 1999. Potential mechanisms of thyroid disruption in humans: interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. *Environmental Health Perspectives* 107(4):273-278

147. Sandau CD, Meerts IATM, Letcher RJ, McLees A, Chittim B, Brouwer A, Norstrom RJ. 2000. Identification of 4-hydroxy-heptachlorostyrene in polar bear plasma and its binding affinity to transthyretin: A possible metabolite of octachlorostyrene? *Environmental Science & Technology* 34(18):3871-3877

148. van den Berg KJ, van Raaij JA, Bragt PC, Notten WR. 1991. Interactions of halogenated industrial chemicals with transthyretin and effects on thyroid hormone levels in vivo. *Archives of Toxicology* 65(1):15-19

149. Dickson PW, Aldred AR, Menting JGT, Marley PD, Sawyer WH, Schreiber G. 1987. Thyroxine transport in choroid plexus. *The Journal of Biological Chemistry* 262(29):13907-13915

150. Santini F, Chopra IJ, Wu S-Y, Solomon DH, Teco GNCT. 1992. Metabolism of 3,5,3'-triiodothyronine sulfate by tissues of the fetal rat: A consideration of the role of desulfation of 3,5,3'-triiodothyronine sulfate as a source of T_3. *Pediatric Research* 31(6):541-544

151. Sandau CD, Ayotte P, Dewailly E, Duffe J, Norstrom RJ. 2000. Analysis of Hydroxylated Metabolites of PCBs (OH-PCBs) and Other Chlorinated Phenolic Compounds in Whole Blood from Canadian Inuit. *Environmental Health Perspectives* 108(7):611-616

152. Schuur AG, Legger FF, van Meeteren ME, Moonen MJ, van Leeuwen-Bol I, Bergman Å, Visser TJ, Brouwer A. 1998. In vitro inhibition of thyroid hormone sulfation by hydroxylated metabolites of halogenated aromatic hydrocarbons. *Chemical Research in Toxicology* 11(9):1075-1081

153. Schuur AG, Brouwer A, Bergman Å, Coughtrie MW, Visser TJ. 1998. Inhibition of thyroid hormone sulfation by hydroxylated metabolites of polychlorinated biphenyls. *Chemico-Biological Interactions* 109(1-3):293-297

154. Schuur AG, van Leeuwen-Bol I, Jong WM, Bergman Å, Coughtrie MW, Brouwer A, Visser TJ. 1998. In vitro inhibition of thyroid hormone sulfation by polychlorobiphenylols: isozyme specificity and inhibition kinetics. *Toxicological Sciences* 45(2):188-194

155. Bastomsky CH, Murthy PV. 1976. Enchanced *in vitro* hepatic glucuronidation of thyroxine in rats following cutaneous application or ingestion of polychlorinated biphenyls. *Canadian Journal of Physiology and Pharmacology* 54(1):23-26

156. Bastomsky CH. 1972. The biliary excretion of thyroxine and its glucuronic acid conjugate in normal and Gunn rats. *Endocrinology* 92(1):35-40

157. Bastomsky CH. 1974. Effects of a polychlorinated biphenyl mixture (Aroclor 1254) and DDT on biliary thyroxine excretion in rats. *Endocrinology* 95(4):1150-1155

158. Bastomsky CH, Murthy PV, Banovac K. 1976. Alterations in thyroxine metabolism produced by cutaneous application of microscope immersion oil: effects due to polychlorinated biphenyls. *Endocrinology* 98(5):1309-1314

159. Saito K, Kaneko H, Sato K, Yoshitake A, Yamada H. 1991. Hepatic UDP-glucuronyltransferase(s) activity toward thyroid hormones in rats: induction and effects on serum thyroid hormone levels following treatment with various enzyme inducers. *Toxicology and Applied Pharmacology* 111(1):99-106

160. Lans MC, Brouwer I, de Winden P, Brouwer A. 1993. Different effects of 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin and Aroclor 1254 on thyroxine metabolism and transport. *Organohalogen Compounds* 13:137-140

161. Visser TJ, Kaptein E, van Toor H, van Raaij JA, van den Berg KJ, Joe CT, van Engelen JG, Brouwer A. 1993. Glucuronidation of thyroid hormone in rat liver: effects of in vivo treatment with microsomal enzyme inducers and in vitro assay conditions. *Endocrinology* 133(5):2177-2186

162. Morse DC, Groen D, Veerman M, Van Amerongen CJ, Koeter HB, Smits vPA, Visser TJ, Koeman JH, Brouwer A. 1993. Interference of polychlorinated biphenyls in hepatic and brain thyroid hormone metabolism in fetal and neonatal rats. *Toxicology and Applied Pharmacology* 122(1):27-33

163. Van Birgelen APJM, van der KJ, Fase KM, Bol I, Poiger H, Brouwer A, van den BM. 1994. Toxic potency of 3,3',4,4',5-pentachlorobiphenyl relative to and in combination with 2,3,7,8-tetrachlorodibenzo-p-dioxin in a subchronic feeding study in the rat. *Toxicology and Applied Pharmacology* 127(2):209-221

164. Van Birgelen APJM, van der Kolk J, Fase KM, Bol I, Poiger H, Brouwer A, van den Berg M. 1994. Toxic potency of 3,3',4,4',5-pentachlorobiphenyl relative to and in combination with 2,3,7,8-tetrachlorodibenzo-p-dioxin in a subchronic feeding study in the rat. *Toxicology and Applied Pharmacology* 127(2):209-221

165. Van Birgelen APJM, Smit EA, Kampen IM, Groeneveld CN, Fase KM, van der Kolk J, Poiger H, van den Berg M, Koeman JH, Brouwer A. 1995. Subchronic effects of 2,3,7,8-TCDD or PCBs on thyroid hormone metabolism: use in risk assessment. *European Journal of Pharmacology* 293(1):77-85

166. Kohn MC, Sewall CH, Lucier GW, Portier CJ. 1996. A mechanistic model on effects of dioxin on thyroid hormones in the rat. *Toxicology and Applied Pharmacology* 165:29-48

167. Adams C, Lans MC, Klasson-Wehler E, van Engelen JGM, Visser TJ, Brouwer A. 1990. Hepatic thyroid hormone 5'-deiodinase, another target-protein for monohydroxy metabolites of 3,3',4,4'-tetrachlorobiphenyl. *Organohalogen Compounds* 1:51-54 (Extended Abstract)

168. Rickenbacher U, Jordan S, McKinney JD. 1989. Structurally specific interaction of halogenated dioxin and biphenyl derivatives with iodothyronine-5'-deiodinase in rat liver. In *Probing Bioactive Mechanisms*, ed. Philip SM, Henry DR, Block JH, Washington, DC: American Chemical Society. 22: pp.354-365.

169. Morse DC, Koeter HBWM, Smits van Prooijen AE, Brower A. 1992. Interference of polychlorinated biphenyls in thyroid hormone metabolism: possible neurotoxic consequences in fetal and neonatal rats. *Chemosphere*:165-168

170. Morse DC, Wehler EK, Wesseling W, Koeman JH, Brouwer A. 1996. Alterations in rat brain thyroid hormone status following pre- and postnatal exposure to polychlorinated biphenyls (Aroclor 1254). *Toxicology and Applied Pharmacology* 136(2):269-279

171. Sinjari T, Darnerud PO. 1998. Hydroxylated polychlorinated biphenyls: placental transfer and effects on thyroxine in the foetal mouse. *Xenobiotica* 28(1):21-30

172. Darnerud PO, Morse D, Klasson-Wehler E, Brouwer A. 1996. Binding of a 3, 3', 4, 4'-tetrachlorobiphenyl (CB-77) metabolite to fetal transthyretin and effects on fetal thyroid hormone levels in mice. *Toxicology* 106:105-114

173. Meerts IATM, Assink Y, Cenijn PH, van den Berg JHJ, Weijers BM, Bergman Å, Koeman JH, Brouwer A. Placental transfer of a hydroxylated polychlorinated biphenyl and effects on fetal and maternal thyroid homeostasis in the rat. 2001. (*in preparation*)

174. van Raaij JA, Frijters CM, Kong LW, van den Berg KJ, Notten WR. 1994. Reduction of thyroxine uptake into cerebrospinal fluid and rat brain by hexachlorobenzene and pentachlorophenol. *Toxicology* 94(1-3):197-208

175. Meerts IATM, Hoving S, van den Berg JHJ, Weijers BM, Swarts HJ, ven der Beek EM, Bergman Å, Koeman JH, Brouwer A. Effects of *in utero* exposure to a hydroxylated polychlorinated biphenyl on developmental landmarks, steroid and thyroid hormone levels, and female estrous cyclicity in rats. 2001. (*in preparation*)

176. Korach KS, Sarver P, Chae K, McLachlan JA, McKinney JD. 1997. Estrogen receptor-binding activity of polychlorinated hydroxybiphenyls:

conformationally restricted structural probes. *Molecular Pharmacology* 33:120-126

177. Klasson-Wehler E, Bergman Å, Brandt I, Darnerund PO, Wachtmeister CA. 1989. 3,3',4,4'-tetrachlorobiphenyl excretion and tissue retention of hydroxylated metabolites in the mouse. *Drug Metabolism and Disposition* 17(4):441-448

178. Wallnofer PR, Engelhardt G, Safe S, Hutzinger O. 1973. Microbial hydroxylation of 4-chlorobiphenyl and 4,4'-dichlorobiphenyl. *Chemosphere* 2:69-72

179. Kramer VJ, Giesy JP, Helferich WT, Bergman Å. 1994. *In vitro* estrogenicity and anti-estrogenicity of hydroxylated chlorinated bipenyls in human breast tumor (MCF-7) cells. *Organohalogen Compounds* 20:475-480

180. Kramer VJ, Helferich WG, Bergman A, Klasson-Wehler E, Giesy JP. 1997. Hydroxylated polychlorinated biphenyl metabolites are anti-estrogenic in a stably transfected human breast adenocarcinoma (MCF7) cell line. *Toxicology and Applied Pharmacology* 144(2):363-376

181. Kramer VJ, Giesy JP. 1999. Specific binding of hydroxylated polychlorinated biphenyl metabolites and other substances to bovine calf uterine estrogen receptor: structure-binding relationships. *The Science of the Total Environment* 233(1-3):141-161

182. Andersson PL, Blom A, Johannisson A, Pesonen M, Tysklind M, Berg AH, Olsson P, Norrgren L. 1999. Assessment of PCBs and Hydroxylated PCBs as Potential Xenoestrogens: In Vitro Studies Based on MCF-7 Cell Proliferation and Induction of Vitellogenin in Primary Culture of Rainbow Trout Hepatocytes. *Archives of Environmental Contamination and Toxicology* 37(2):145-150

183. Connor K, Ramamoorthy K, Moore M, Mustain M, Chen I, Safe S, Zacharewski T, Gillesby B, Joyeux A, Balaguer P. 1997. Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: structure-activity relationships. *Toxicology and Applied Pharmacology* 145(1):111-123

184. Moore M, Mustain M, Daniel K, Chen I, Safe S, Zacharewski T, Gillesby B, Joyeux A, Balaguer P. 1997. Antiestrogenic activity of hydroxylated polychlorinated biphenyl congeners identified in human serum. *Toxicology and Applied Pharmacology* 142:160-168

185. Edmunds JSG, Fairey ER, Ramsdell JS. 1997. A rapid and sensitive high throughput reporter gene assay for estrogenic effects of environmental contaminants. *Neurotoxicology.* 18(2):525-532

186. Ramamoorthy K, Vyhlidal C, Chen I-C, Safe S, McDonnell DP, Leonard LS, Gaido KW. 1997. Additive estrogenic activities of a binary mixture of 2', 4', 6'-trichloro- and 2', 3', 4', 5'-tetrachloro-4-biphenylol. *Toxicology and Applied Pharmacology* 147:93-100

187. Matthews J, Zacharewski T. 2000. Differential binding affinities of PCBs, HO-PCBs, and aroclors with recombinant human, rainbow trout (Onchorhynkiss mykiss), and green anole (Anolis carolinensis) estrogen receptors, using a semi-high throughput competitive binding assay. *Toxicological Sciences* 53(2):326-339

188. Vakharia DD, Gierthy JF. 2000. Use of a combined human liver microsome-estrogen receptor binding assay to assess potential estrogen modulating activity of PCB metabolites. *Toxicology Letters* 114(1-3):55-65

189. Kuiper GGJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JÅ. 2000. Interation of estrogenic chemicals and phytoestrogens with estrogen receptor β . *Endocrinology* 139(10):4252-4263

190. Kester MHA, Bulduk S, Tibboel D, Meinl W, Glatt H, Falany CN, Coughtrie MWH, Bergman Å, Safe SH, Kuiper GGJM, Schuur AG, Brouwer A, Visser TJ. 2000. Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: a novel pathway explaining the estrogenic activity of PCBs. *Endocrinology* 141(5):1897-1900

191. Klasson-Wehler E, Bergman Å, Athanasiadou M, Ludwig JP, Auman HJ, Kannan K, van den Berg M, Murk AJ, Feyk LA, Giesy JP. 1998. Hydroxylated and methylsulfonyl polychlorinated biphenyl metabolites in albatrosses from Midway Atoll, North Pacific Ocean. *Environmental Toxicology and Chemistry* 17(8):1620-1625

192. Sjödin A, Hagmar L, Klasson-Wehler E, Björk J, Bergman Å. 2000. Neutral and phenolic organohalogen contaminants in human plasma related to intake of Baltic fish. *Environmental Health Perspectives* 108(11):1035-1041.

193. De Haan LHJ, Van Dijk L, Brouwer A. 1994. Effects of hydroxylated PCB metabolites on the gap-junctional intercellular communication between mouse hepa1c1c7 cells. *Organohalogen Compounds* 20:491-495

194. Kolaja KL, Engelken DT, Klaassen CD. 2000. Inhibition of gap-junctional-intercellular communication in intact rat liver by nongenotoxic hepatocarcinogens. *Toxicology* 146(1):15-22

195. Nishihara Y. 1988. Comparative toxicity of 4-chlorobiphenyl and its metabolite 4-chloro-4'-biphenylol in isolated rat liver mitochondria. *Biochemical Pharmacology* 37(15):2915-2926

196. Ebner KV, Braselton WEJ. 1987. Structural and chemical requirements for hydroxychlorobiphenyls to uncouple rat liver mitochondria and potentiation of uncoupling with aroclor 1254. *Chemico-Biological Interactions* 63(2):139-155

197. Lans MC, Kubiczak G, Douwes J, Brouwer A. 1990. *In vitro* effects of 3,4,3',4'-tetrachlorobipenyl and its hydroxymetabolites on mitochondrial function. *Organohalogen Compounds* 1:103-106

198. Narasimhan TR, Kim HL, Safe SH. 1991. Effects of hydroxylated polychlorinated biphenyls on mouse liver mitochondrial oxidative phosphorylation. *Journal of Biochemical Toxicology* 6(3):229-236

199. Amaro AR, Oakley GG, Bauer U, Spielmann HP, Robertson LW. 1996. Metabolic activation of PCBs to quinones: reactivity toward nitrogen and sulfur nucleophiles and influence of superoxide dismutase. *Chemical Research in Toxicology* 9(3):623-629

200. Jansson B, Jensen S, Olsson M, Renberg L, Sundstrom G, Vaz R. 1975. Identification by GC-MS of phenolic metabolites of PCB and p,p'-DDE isolated from Baltic guillemot and seal. *Ambio*:93-97

201. Klasson-Wehler E, Hovander L, Bergman Å. 1997. New organohalogens in human plasma - Identification and quantification. *Organohalogen Compounds* 33:420-425 (Extended Abstract)

202. Bergman Å, Brouwer A, Ghosh M, Hagmar L, Jakobsson E, Johnson L, Klasson-Wehler E, Meerts I, Sauer PJJ, Weisglas-Kuperus N. 1997. Risk of endocrine contaminants (RENCO) - Aims and a summary of initial results. *Organohalogen Compounds* 34:396-401 (Extended Abstract)

203. Sandau CD, Norstrom RJ. 1996. Comparison of methylation methods for the determination of hydroxy-PCBs and preliminary results for polar bear blood plasma. *Organohalogen Compounds* 29:412-417 (Extended Abstract)

204. Sandau CD, Ramsay MA, Norstrom RJ. Implications of hydroxylated metabolites of PCBs and other halogenated phenolic compounds as endocrine disruptors in polar bears. Clement, R. and Burk, R. 247-252. 2000. Proceedings of the Third Biennial International Conference on Chemical Measurement and Monitoring of the Environment (Enviroanalysis 2000). (Extended Abstract)

205. Olsson A, Ceder K, Bergman Å, Helander B. 2000. Nestling blood of the white-tailed sea eagle (*Haliaeetus albicilla*) as an indicator of territorial exposure to organohalogen compounds - An evaluation. *Environmental Science & Technology* 34(13):2733-2740

206. Newson SC, Sandau CD, Elliott JE, Brown SB, Norstrom RJ. PCBs and hydroxylated metabolites in bald eagle plasma - comparison to thyroid hormone and retinol levels. Clement, R. and Burk, R. 257-262. 2000. Proceedings of the Third Biennial International Conference on Chemical Measurement and Monitoring of the Environment (Enviroanalysis 2000). (Extended abstract)

207. Asplund L, Athanasiadou M, Sjödin A, Bergman Å, Börjeson H. 1999. Organohalogen substances in muscle, egg and blood from healthy Baltic salmon (*Salmo salar*) and Baltic salmon that produced offspring with the M74 syndrome. *Ambio* 28(1):67-76

208. Zitko V, Hutzinger O, Choi PM. 1974. Determination of pentachlorophenol and chlorobiphenylols in biological samples. *Bulletin of Environmental Contamination & Toxicology* 12(6):649-653

209. Newsome WH, Davies D. 1996. Determination of PCB metabolites in Canadian human milk. *Chemosphere.* 33(3):559-565

210. Hassanzadeh P, Guvenius DM, Norén K. 2000. Hydroxylated metabolites of polychlorinated biphenyls (OH-PCBs) in human liver and adipose tissue. *Organohalogen Compounds* 48:54-57 (Extended Abstract)

211. Bergman Å, Klasson-Wehler E, Kuroki H, Nilsson A. 1995. Synthesis and mass spectrometry of some methoxylated PCB. *Chemosphere* 30(10):1921-1938

212. Safe S, Washburn K, Zacharewski T, Phillips T. 1995. Synthesis and characterization of hydroxylated polychlorinated biphenyls (PCBs) identified in human serum. *Chemosphere* 31(4):3017-3023

213. Bauer U, Amaro AR, Robertson LW. 1995. A new strategy for the synthesis of polychlorinated biphenyl metabolites. *Chemical Research in Toxicology* 8(1):92-95

214. Lötjönen S, Äyräs P, Pyysalo H. 1979. Glass capillary gas chromatographic detection and mass spectral analysis of some hydroxyl derivatives of polychlorinated biphenyls (PCB's). *Finnish Chemical Letters*:57-60

215. Rozemeijer MJC, Olie K, de Voogt P. 1997. Procedures for analysing phenolic metabolites of polychlorinated dibenzofurans, -dibenzo-p-dioxins and -biphenyls extracted from a microsomal assay: optimising solid-phase adsorption clean-up and derivatisation methods. *Journal of Chromatography A* 761:219-230

216. Hopper ML. 1987. Methylation of chlorophenoxy acid herbicides and pentachlorophenol residues in foods using ion-pair alkylation. *Journal of Agricultural and Food Chemistry* 35:265-269

217. Stalling DL, Hogan JW. 1978. Preparation, separation and identification of TMS derivatives of hydroxylated PCBS and chlorophenols. *Bulletin of Environmental Contamination & Toxicology* 20(1):35-43

218. Abraham VM, Lynn BCJ. 1997. Determination of hydroxylated polychlorinated biphenyls by ion trap gas chromatography-tandem mass spectrometry. *Journal of Chromatography A* 790(1-2):131-141

219. Hovander L, Athanasiadou M, Asplund L, Jensen S, Klasson Wehler E. 2000. Extraction and cleanup methods for analysis of phenolic and neutral organohalogens in plasma. *Journal of Analytical Toxicology* 24(8):696-703

220. Hovander L, Athanasiadou M, Asplund L, Klasson-Wehler E. 1998. A method of analysis of phenolic and neutral organic halogenated substances in plasma. *Organohalogen Compounds* 35:115-118 (Extended Abstract)

221. Blatt AH. 1943. *Organic Syntheses - Collective Volume 2.* New York: John Wiley & Sons, Inc. pp.1-564

222. Stemmler EA, Hites RA, Arbogast B, Budde WL, Deinzer ML, Dougherty RC, Eichelberger JW, Foltz RL, Grimm C, Grimsrud EP, Sakashita C, Sears LJ. 1988. Interlaboratory comparison of methane electron capture negative ion mass spectra. *Analytical Chemistry* 60:781-787

223. Crow FW, Bjorseth A, Knapp KT, Bennett R. 1981. Determination of polyhalogenated hydrocarbons by glass capillary gas chromatography-negative ion chemical ionization mass spectrometry. *Analytical Chemistry* 53:619-625

224. Stemmler EA, Hites RA. 1985. Methane enhanced negative ion mass spectra of hexachlorocyclopentadiene derivatives. *Analytical Chemistry* 57:684-692

225. Stemmler EA, Hites RA. 1988. A systematic study of instrumental parameters affecting electron capture negative ion mass spectra. *Biomedical and Environmental Mass Spectrometry* 15:659-667

226. Haraguchi K, Bergman A, Jakobsson E, Masuda Y. 1993. Negative ion chemical ionization mass spectrometry in the analysis of polychlorinated biphenyl methyl sulphones. *Fresenius' Zeitschrift für Analytische Chemie* 347:441-449

227. Letcher RJ, Norstrom RJ. 1997. Electron-capture/negative ion mass spectrometric characteristics of bioaccumulating methyl sulfone-substituted polychlorinated biphenyls. *Journal of Mass Spectrometry* 32(2):232-240

228. Sears LJ, Campbell JA, Grimsrud EP. 1987. Ionization dynamics within the high pressure electron capture mass spectrometer; the unusual spectra of derivatized polycyclic aromatic amines and perchlorinated unsaturated hydrocarbons. *Biomedical and Environmental Mass Spectrometry* 14:401-415

229. Oehme M, Stöckel D, Knöppel H. 1986. Comparison of the reproducibility of negative ion chemical ionization mass spectra obtained by different reagent gases on two commercial quadrupole instruments. *Analytical Chemistry* 58:554-558

230. Letcher RJ, Norstrom RJ, Muir DCG. 1998. Biotransformation versus bioaccumulation: Sources of methyl sulfone PCB and 4,4'-DDE metabolites in the polar bear food chain. *Environmental Science & Technology* 32:1656-1661

231. Letcher RJ, Klasson-Wehler E, Bergman Å. 1999. Methyl sulfone and hydroxylated metabolites of polychlorinated biphenyls. In *The Handbook of Environmental Chemistry - New Types of Persistent Halogenated Compounds*, ed. Paasivirta J, Berlin: Springer-Verlag. 11: pp.317-359

232. Burse VW, Moseman RF, Sovocool GW, Villanueva EC. 1976. PCB metabolism in rats following prolonged exposure to Aroclor 1242 and aroclor 1016. *Bulletin of Environmental Contamination & Toxicology* 15(1):122-128

233. Gardner AM, Righter HF, Roach JA. 1976. Excretion of hydroxylated polychlorinated biphenyl metabolites in cows' milk. *Journal Association of Official Analytical Chemists* 59(2):273-277

234. Safe S, Platonow N, Hutzinger O. 1975. Metabolism of chlorobiphenyls in the goat and cow. *Journal of Agricultural and Food Chemistry* 23(2):259-261

235. Norstrom RJ, Belikov SE, Born EW, Garner GW, Malone B, Olpinski S, Ramsay MA, Schliebe S, Stirling I, Stishov MS, Taylor MK, Wiig O. 1998. Chlorinated hydrocarbon contaminants in polar bears from eastern Russia, North America, Greenland, and Svalbard: biomonitoring of Arctic pollution. *Archives of Environmental Contamination and Toxicology* 35(2):354-367

236. Yieru H, Qingyu O, Weile Y. 1990. Characteristics of flame ionization detection for the quantitative analysis of complex organic mixtures. *Analytical Chemistry* 62:2063-2064

237. Sperry WM. 1963. Gravimetric determination of total lipids in blood serum or plasma. *Standard Methods in Clinical Chemistry* 4:173-182

238. Jacobs SL, Henry RJ. 1962. Studies on the gravimetric determination of serum lipids. *Clinica Chimica ACTA* 7:270-276

239. Chabrol É, Charonnat R. 1937. Une nouvelle réaction pour l'étude des lipides: l'oleidemie. *La Presse Medicale* 96:1713-1714

240. Frings CS, Dunn RT. 1970. A colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reaction. *American Journal of Clinical Pathology* 53:89-91

241. Frings CS, Fendley TW, Dunn RT, Queen CE. 1972. Improved determination of total serum lipids by the sulfo-phospho-vanillin reaction. *Clinical Chemistry* 18(7):673-674

242. Knight JA, Anderson S, Rawle JM. 1972. Chemical basis of the sulfo-phospho-vanillin reaction for estimating total serum lipids. *Clinical Chemistry* 18(3):199-202

243. Matthews HB, Surles JR, Carver JG, Anderson MW. 1984. Halogenated biphenyl transport by blood components. *Fundamental and Applied Toxicology* 4(3 Pt 1):420-428

244. Mes J, Marchand L, Turton D, Lau P-Y. 1992. The determination of polychlorinated biphenyl congeners and other chlorinated hydrocarbon residues in human blood, serum and plasma. A comparitive study. *International Journal of Environmental Analytical Chemistry* 48:176-186

245. Hebert CE, Keenleyside KA. 1995. To normalize or not to normalize? Fat is the question. *Environmental Toxicology and Chemistry* 14(5):801-807

246. Rickenbacher U, McKinney JD, Oatley SJ, Blake CCF. 1986. Structurally specific binding of halogenated biphenyls to thyroxine transport protein. *Journal of Medicinal Chemistry* 29:641-648

247. Köhrle J. 1999. Local activation and inactivation of thyroid hormones: the deiodinase family. *Molecular and Cellular Endocrinology* 151:103-119

248. Marsh G, Bergman Å, Bladh L-G, Gillner M, Jakobsson E. 1998. Synthesis of *p*-hydroxybromodiphenyl ethers (OH-PBDEs) and binding to the thyroid hormone receptor. *Organohalogen Compounds* 37:305-308 (Extended Abstract)

249. Chu I, Villeneuve DC, Secours V, Benoit FM, Viau A. 1982. The tissue distribution, metabolism, and excretion of octachlorostyrene in the rat. *Drug Metabolism and Disposition* 10(6):632-635

250. Norstrom RJ, Muir DCG. 1994. Chlorinated hydrocarbon contaminants in arctic marine mammals. *The Science of the Total Environment* 154:107-128

251. Letcher RJ, Norstrom RJ, Bergman A. 1995. Geographical distribution and identification of methyl sulphone PCB and DDE metabolites in pooled polar bear (Ursus maritimus) adipose tissue from western hemisphere arctic and subarctic regions. *The Science of the Total Environment* 160-161:409-420

252. Letcher RJ, Norstrom RJ, Lin S, Ramsay MA, Bandiera SM. 1996. Immunoquantitation and microsomal monooxygenase activities of hepatic cytochromes P4501A and P4502B and chlorinated hydrocarbon contaminant levels in polar bear (Ursus maritimus). *Toxicology and Applied Pharmacology* 137(2):127-140

253. Letcher RJ, Norstrom RJ, Bergman Å. 1995. An integrated analytical method for determination of polychlorinated aryl methyl sulfone metabolites and polychlorinated hydrocarbon contaminants in biological matrices. *Analytical Chemistry* 67(22):4155-4163

254. Tittlemier SA, Simon M, Jarman WM, Elliott JE, Norstrom RJ. 1999. Identification of a novel $C_{10}H_6N_2Br_4Cl_2$ heterocyclic compound in seabird eggs. A bioaccumulating marine natural product? *Environmental Science & Technology* 33(1):26-33

255. Jansson B, Sundstrom G. 1974. Mass spectrometry of the methyl ethers of isomeric hydroxychlorobiphenyls - potential metabolites of chlorobiphenyls. *Biomedical Mass Spectrometry* 1:386-392

256. Pring BG, Stjernstrom NE. 1968. Complex dibenzofurans XII. The use of oxygen-18 labelling in the interpretation of mass spectra of some biphenyl and dibenzofuran derivatives. *Acta Chemica Scandinavica* 22(2):549-561

257. Lau B, Weber D, Andrews P. 1996. GC-MS analysis of toxaphene: a comparative study of different mass spectrometric techniques. *Chemosphere* 32(6):1021-1041 (Extended Abstract)

258. Lans MC, Klasson-Wehler E, Brouwer A. 1994. Thyroid hormone binding proteins as targets for hydroxylated PCB, PCDD and PCDF metabolites; an overview. *Organohalogen Compounds* 20:481-485

259. Shiu W-Y, Ma K-C, Varhanícková D, Mackay D. 1994. Chlorophenols and alkylphenols: A review and correlation of environmentally relevant properties and fate in an evaluative environment. *Chemosphere* 29(6):1155-1224

260. Norstrom RJ, Letcher RJ. 1997. Role of Biotransformation in Bioconcentration and Bioaccumulation. In *Biotransformation in Environmental Risk Assessment*, ed. Sijm D, de Bruijn J, de Voogt P, de Wolf W, Brussels: SETAC-Europe. pp.101-113.

261. Bester K, Biselli S, Ellerichmann T, Huhnerfuss H, Moller K, Rimkus G, Wolf M. 1998. Chlorostyrenes in fish and sediment samples from the river Elbe. *Chemosphere* 37(9-12):2459-2471

262. Kuehl DW, Kopperman HL, Veith GD, Glass GE. 1976. Isolation and identification of polychlorinated styrenes in Great Lakes fish. *Bulletin of Environmental Contamination & Toxicology* 16(2):127-132

263. van Ommen B, Hendriks W, Bessems JGM, Geesink G, Van Bladeren PJ. 1989. The relation between the oxidative biotransformation of hexachlorobenzene and its porphyrinogenic action. *Toxicology and Applied Pharmacology* 100:517-528

264. Weis IM, Muir DCG. 1997. Geographical variation of persistent organochlorine concentrations in blubber of ringed seal (*Phoca Hispida*) from the Canadian arctic: Univariate and multivariate approaches. *Environmental Pollution* 96(3):321-333

265. Muir D, Riget F, Cleeman M, Skaare J, Kleivane L, Nakata H, Dietz R, Severinsen T, Tanabe S. 2000. Circumpolar trends of PCBs and organochlorine pesticides in the Arctic marine environment inferred from levels in ringed seals. *Environmental Science & Technology* 34(12):2431-2438

266. Kaminsky R, Hites RA. 1984. Octachlorostyrene in Lake Ontario: Sources and fate. *Environmental Science & Technology* 18(4):275-279

267. Vogelgesang J, Kypke-Hutter K, Malisch R, Binnemann P, Dietz W. 1986. The origin of a contamination of fish from the river Neckar with hexachlorobenzene, octachlorostyrene and pentachlorobenzene: Formation in an industrial process. *Zeitschrift Fur Lebensmittel -Unterschung und - Forschung* 182:471-474

268. Ofstad EB, Lunde G, Martinsen K, Rygg B. 1978. Chlorinated aromatic hydrocarbons in fish from an area polluted by industrial effluents. *The Science of the Total Environment* 10(3):219-230

269. van den Brink NW, de Rulter-Dukman EM. 1997. Trans-nonachlor, octachlorostyrene, mirex and photomirex in Antarctic seabirds. *Antarctic Science* 9(4):414-417

270. Stirling I, McEwan EH. 1975. The caloric value of whole ringed seals (*Phoca hispida*) in relation to polar bear (*Ursus maritimus*) ecology and hunting behaviour. *Canadian Journal of Zoology* 53(1021):1027

271. Leatherland JF, Ronald K. 1981. Plasma concentrations of thyroid hormones in a captive and feral polar bear (Ursus maritimus). *Comparative Biochemistry and Physiology* 70(4):575-577

272. Skaare JU, Bernhoft A, Wiig Ø, Norum KR, Haug E, Eide DM, Derocher AE. 2001. Relationships between plasma levels of organochlorines, retinol and thyroid hormones from polar bears (*Ursus maritimus*) at Svalbard. *Journal of Toxicology and Environmental Health* (in press)

273. Muir DC, Wagemann R, Hargrave BT, Thomas DJ, Peakall DB, Norstrom RJ. 1992. Arctic marine ecosystem contamination. *The Science of the Total Environment* 122(1-2):75-134

274. Norheim G, Skaare JU, Wiig Ø. 1992. Some heavy metals, essential elements and chlorinated hydrocarbons in polar bear (*Ursus maritimus*) at Svalbard. *Environmental Pollution* 77:51-57

275. Calvert W, Ramsay MA. 1998. Evaluation of age determination of polar bears by counts of cementum growth layer groups. *Ursus* 10:449-453

276. Honour SM, Trudeau S, Kennedy S, Wobeser G. 1995. Experimental vitamin A deficiency in mallards (*Anas platyrhynchos*): lesions and tissue vitamin A levels. *Journal of Wildlife Diseases* 31(3):277-288

277. Eales JG, Shostak, S. 1985. Free T_4 and T_3 relation to total hormone, free hormone indices, and protein in plasma of Rainbow trout and Arctic charr. *General and Comparative Endocrinology* 58:291-302

278. Bernhoft A, Wiig O, Skaare JU. 1997. Organochlorines in polar bears (*Ursus maritimus*) in Svalbard. *Environmental Pollution* 95(2):159-175

279. Muir DCG, Norstrom RJ. 2000. Geographical differences and time trends of persistent organic pollutants in the Arctic. *Toxicology Letters* 112-113:93-101

280. Polischuk SC, Letcher RJ, Norstrom RJ, Ramsay MA. 1995. Preliminary results of fasting on the kinetics of organochlorines in polar bears (Ursus maritimus). *The Science of the Total Environment* 160-161:465-472

281. Polischuk SC, Norstrom RJ, Ramsay MA. Body burdens and tissue concentrations of organochlorines in polar bears (*Ursus maritimus*) vary during seasonal fasts. 2001. (*in preparation*)

282. Skaare JU, Tuveng JM, Sande HA. 1988. Organochlorine pesticides and polychlorinated biphenyls in maternal adipose tissue, blood, milk, and cord blood from mothers and their infants living in Norway. *Archives of Environmental Contamination and Toxicology* 17:55-63

283. Cattell R. 1966. The meaning and strategic use of factor analysis. In *Handbook of multivariate experimental psychology*, ed. Cattell RB, Chicago: Rand McNally. pp.174-243

284. Lindblad BS, Patel M, Hamadeh M, Helmy N, Ahmad I, Dawodu A, Zaman S. 1998. Age and sex are important factors in determining normal retinol levels. *Journal of Tropical Pediatrics* 44:96-99

285. Chen L-C, Berberian I, Koch B, Mercier M, Azalis-Braesco V, Glauert HP, Chow CK, Robertson LW. 1992. Polychlorinated and polybrominated biphenyl congeners and retinoid levels in rat tissues: structure-activity relationships. *Toxicology and Applied Pharmacology* 114:47-55

286. Skåre JU, Wiig Ø, Bernhoft A. 1994. *Klorerte organiske miljøgifter; nivåer og effekter på isbjørn. Rep. 86*, Norsk Polarinstitutt, Norway

287. Goldey ES, Kehn LS, Lau C, Rehnberg GL, Crofton KM. 1995. Developmental exposure to polychlorinated biphenyls (Aroclor 1254) reduces circulating thyroid hormone concentrations and causes hearing deficits in rats. *Toxicology and Applied Pharmacology* 135(1):77-88

288. Guillette LJ, Jr. 2000. (Personal Communication)

289. Jensen S. 1972. The PCB story. *Ambio*:123-131

290. Schnellman RG, Putnam CW, Sipes IG. 1983. Metabolism of 2,3', 3,3', 6,6'-hexachlorobiphenyl and 2,2', 4, 4', 5,5'- hexachlorobiphenyl by human hepatic microsomes. *Biochemical Pharmacology* 32:3233-3239

291. Caldwell J. 1986. *Xenobiotic conjugation chemistry*. Washington D.C.: American Chemical Society. pp.2-28

292. Brouwer A, Klasson-Wehler E, Bokdam MM. 1990. Competitive inhibition of thyroxin binding to transthyretin by monohydroxy metabolites of 3,4,3'4'-tetrachlorobiphenyl. *Chemosphere* 20:1257-1262

293. Kuhnlein HV, Soueida R, Receveur O. 1995. Baffin Inuit food use by age, gender and season. *Journal of The Canadian Dietetic Association* 56(4):175-183

294. Kuhnlein HV, Kubow S, Soueida R. 1991. Lipid components of traditional inuit foods and diets of Baffin Island. *Journal of Food Composition and Analysis* 4:227-236

295. Kuhnlein HV. 1995. Benefits and risks of traditional food for Indigenous Peoples: focus on dietary in takes of Arctic men. *Canadian Journal of Physiology and Pharmacology* 73:765-771

296. Cameron M, Weis IM. 1993. Organochlorine contaminants in the country food diet of the Belcher Island Inuit, Northwest Territories, Canada. *Arctic* 46:42-48

297. Stern GA, Muir DCG, Segstro M, Dietz R, Heide-Jorgenson M. 1994. PCBs and other organochlorine contaminants in white whales (*Delphinapterus leucas*) from West Greenland: variations with age and sex. *Meddelelser om Grønland, Bioscience* 39:245-259

298. Newsome WH, Davies DJ, Sun WF. 1998. Residues of polychlorinated biphenyls (PCB) in fatty foods of the Canadian diet. *Food Additives and Contaminants* 15(1):19-29

299. Ayotte P, Dewailly E, Ryan JJ, Bruneau S, LeBel G. 1997. PCBs and dioxin-like compounds in plasma of adult Inuit living in Nunavik (Arctic Quebec). *Chemosphere* 34(5-7):1459-1468

300. Seegal RF. 1996. Epidemiological and laboratory evidence of PCB-induced neurotoxicity. *Critical Reviews in Toxicology* 26(6):709-737

301. Geyer HJ, Scheunert I, Korte F. 1987. Distribution and bioconcentration of the environmental chemical pentachlorophenol (PCP) in different tissues of humans. *Chemosphere* 16(4):887-899

302. Dewailly E, Nantel A, Weber JP, Meyer F. 1989. High levels of PCBs in breast milk of Inuit women from arctic Quebec. *Bulletin of Environmental Contamination & Toxicology* 43(5):641-646

303. Dewailly E, Weber J-P, Gingras S, Laliberté C. 1991. Coplanar PCBs in human milk in the province of Québec, Canada: Are they more toxic than dioxin for breast fed infants? *Bulletin of Environmental Contamination & Toxicology* 47:491-498

304. Dewailly E, Ayotte P, Laliberté C, Weber J-P, Gingras S, Nantel AJ. 1996. Polychlorinated biphenyls (PCBs) and dichlorodiphenyl dichloroethylene (DDE) in breast milk of women living in the province of Quebec, Canada. *American Journal of Public Health* 86(9):1241-1246

305. Silverman DI, Reis GJ, Sacks FM, Boucher TM, Pasternak RC. 1990. Usefulness of plasma phospholipid N-3 fatty acid levels in predicting dietary fish intake in patients with coronary artery disease. *American Journal of Cardiology* 66(10):860-862

306. Fein GG, Jacobson JL, Jacobson SW, Schwartz PM, Dowler JK. 1984. Prenatal exposure to polychlorinated biphenyls: effects on birth size and gestational age. *The Journal of Pediatrics* 105(2):315-320

307. Jacobson SW, Fein GG, Jacobson JL, Schwartz PM, Dowler JK. 1985. The effect of intrauterine PCB exposure on visual recognition memory. *Child Development* 56(4):853-860

308. Jacobson JL, Jacobson SW, Humphrey HE. 1990. Effects of in utero exposure to polychlorinated biphenyls and related contaminants on cognitive functioning in young children. *The Journal of Pediatrics* 116(1):38-45

309. Jacobson JL, Jacobson SW. 1997. Evidence for PCBs as neurodevelopmental toxicants in humans. *Neurotoxicology.* 18(2):415-424

310. Porterfield SP, Hendry LB. 1998. Impact of PCBs on thyroid hormone directed brain development. *Toxicology and Environmental Health* 14(1-2):103-120

311. Hamburgh M. 1969. The role of thyroid and growth hormones in neurogenesis. In *Current Topics in Developmental Biology*, ed. Moscona A, Monroy A, New York: Academic Press. pp.109-148

312. Koopman-Esseboom C, Morse DC, Weisglas-Kuperus N, Lutkesschipholt IJ, Van der Paauw CG, Tuinstra LGMT, Brouwer A, Sauer PJJ. 1994. Effects of dioxins and polychlorinated biphenyls on thyroid hormone status of pregnant women and their infants. *Pediatric Research* 36(4):468-473

313. Koopman-Esseboom C, Huisman M, Weisglas-Kuperus N, Van der Paauw CG, Tuinstra LGMTh, Boersma ER, Sauer PJJ. 1994. PCB and dioxin levels in plasma and human milk of 418 dutch women and their infants. Predictive value of PCB congener levels in maternal plasma for fetal and infant's exposure to PCBs and dioxins. *Chemosphere* 28(9):1721-1732

314. Huisman M, Koopman-Esseboom C, Fidler V, Hadders-Algra M, Van der Paauw CG, Tuinstra LGM, Weisglas-Kuperus N, Sauer PJJ, Touwen BCL, Boersma ER. 1995. Perinatal exposure to polychlorinated biphenyls and dioxins and its effect on neonatal neurological development. *Early Human Development* 41:111-127

315. Muckle G, Dewailly E, Ayotte P. 1998. Prenatal exposure of Canadian children to polychlorinated biphenyls and mercury. *Canadian Journal of Public Health* 89 Suppl 1:S20-5, 22-7:S20-S27

316. Rice DC, Hayward S. 1999. Effects of postnatal exposure of monkeys to a PCB mixture on concurrent random interval-random interval and progressive ratio performance. *Neurotoxicology and Teratology* 21(1):47-58

317. Dewailly E, Ayotte P, Bruneau S, Laliberte C, Muir DCG, Norstrom RJ. 1993. Inuit exposure to organochlorines through the aquatic food chain in Arctic Quebec. *Environmental Health Perspectives* 101:618-620

318. Dewailly E, Laliberté C, Sauvé L, Ferron L, Ryan JJ, Gingras S, Ayotte P. 1992. Sea-bird egg consumption as a major source of PCB exposure for communities living along the Gulf of St.Lawrence. *Chemosphere* 25(7-10):1251-1255

319. Uhl S, Schmid P, Schlatter C. 1986. Pharmacokinetics of pentachlorophenol in man. *Archives of Toxicology* 58(3):182-186

320. World Health Organization 1987. *Pentachlorophenol - Environmental Health Criteria 71*. Geneva: World Health Organization. pp.3-236

321. Wild SR, Jones KC. 1992. Pentachlorophenol in the UK environment II: Human exposure and an assessment of pathways. *Chemosphere* 24(7):847-855

322. Daniel V, Huber W, Bauer K, Opelz G. 1995. Impaired in-vitro lymphocyte responses in patients with elevated pentachlorophenol (PCP) blood levels. *Archives of Environmental Health* 50(4):287-292

323. Waidyanatha S, Lin PH, Rappaport SM. 1996. Characterization of chlorinated adducts of hemoglobin and albumin following administration of pentachlorophenol to rats. *Chemical Research in Toxicology* 9(3):647-653

324. Lin PH, Waidyanatha S, Rappaport SM. 1996. Investigation of liver binding of pentachlorophenol based upon measurement of protein adducts. *Biomarkers* 1:232-243

325. Beard AP, Bartlewski PM, Chandolia RK, Honaramooz A, Rawlings NC. 1999. Reproductive and endocrine function in rams exposed to the organochlorine pesticides lindane and pentachlorophenol from conception. *Journal of Reproduction and Fertility* 115(2):303-314

326. Schuur AG, Bergman Å, Brouwer A, Visser TJ. 1999. Effects of pentachlorophenol and hydroxylated polychlorinated biphenyls on thyroid hormone conjugation in a rat and human hepatoma cell line. *Toxicology In Vitro* 13:417-425

327. Jain SK, Shah M, Ransonet L, Wise R, Bocchini JAJ. 1995. Maternal and neonatal plasma transthyretin (prealbumin) concentrations and birth weight of newborn infants. *Biology of the Neonate* 68(1):10-14

328. Ando M, Saito H, Wakisaka I. 1985. Transfer of polychlorinated biphenyls (PCBs) to newborn infants through the placenta and mothers' milk. *Archives of Environmental Contamination and Toxicology* 14(1):51-57

329. Hanzlik RP, Hogberg K, Judson CM. 1984. Microsomal hydroxylation of specifically deuterated monosubstituted benzenes. Evidence for direct aromatic hydroxylation. *Biochemistry* 23:3048-3055

330. Selander HG, Jerina DM, Piccolo DE, Berchtold GA. 1975. Synthesis of 3- and 4-chlorobenzene oxides. Unexpected trapping results during metabolism of [^{14}C]chlorobenzene by hepatic microsomes. *Journal of the American Chemical Society* 97(15):4428-4430

331. Selander HG, Jerina DM, Daly JW. 1975. Metabolism of chlorobenzene with hepatic microsomes and solubilized cytochrome P-450 systems. *Archives of Biochemistry and Biophysics* 168:309-321

332. Preston BD, Miller JA, Miller EC. 1984. Reactions of 2,2',5,5'-tetrachlorobiphenyl 3,4-oxide with methionine, cysteine and glutathione in relation to the formation of methylthio-metabolites of 2,2',5,5'-tetrachlorobiphenyl in the rat and mouse. *Chemico-Biological Interactions* 50:289-312

333. Preston BD, Allen JR. 1980. 2,2',5,5'-Tetrachlorobiphenyl: isolation and identification of metabolites generated by rat liver microsomes. *Drug Metabolism and Disposition* 8(4):197-204

334. Drouillard, KG, Norstrom RJ. 2001. Evaluation of gastrointestinal magnification in ring doves (*Streptopelia risoria*) fed a polychlorinated biphenyl contaminated diet (*in preparation*)

335. Tanabe Y, Ishii T, Tamaki Y. 1969. Comparison of thyroxine-binding plasma proteins of various vertebrates and their evolutionary aspects. *General and Comparative Endocrinology* 13:14-21

336. Beard AP, Bartlewski PM, Rawlings NC. 1999. Endocrine and reproductive function in ewes exposed to the organochlorine pesticides lindane or pentachlorophenol. *Journal of Toxicology and Environmental Health* 56(1):23-46

337. Beard AP, Rawlings NC. 1999. Thyroid function and effects on reproduction in ewes exposed to the organochlorine pesticides lindane or pentachlorophenol (PCP) from conception. *Journal of Toxicology and Environmental Health* 58(1):509-530

338. Jekat FW, Meisel ML, Eckard R, Winterhoff H. 1994. Effects of pentachlorophenol (PCP) on the pituitary and thyroidal hormone regulation in the rat. *Toxicology Letters* 71(1):9-25

339. Porterfield SP. 1994. Vulnerability of the developing brain to thyroid abnormalities: environmental insults to the thyroid system. *Environmental Health Perspectives* 102 Suppl 2:125-30:125-130

340. Lu M-H, Anderson RR. 1994. Thyroxine secretion rates during pregnancy in the rat. *Endocrine Research* 20(4):343-364

341. Porterfield SP, Hendrich CE. 1993. The role of thyroid hormones in prenatal and neonatal neurological development--current perspectives. *Endocrine Reviews* 14(1):94-106

Appendix Table 1 - PCB numbering scheme as described by Ballschmiter and Zell (1) with modifications proposed by Guitart *et al.* (2) (shaded congeners).

No.	Structure	Ring
Monochlorobiphenyls		
1	2	2
2	3	3
3	4	4
Dichlorobiphenyls		
4	2,2'	2 - 2
5	2,3	2,3
6	2,3'	2 - 3'
7	2,4'	2 - 4'
8	2,4	2,4
9	2,5	2,5
10	2,6	2,6
11	3,3'	3, 3'
12	3,4	3,4
13	3,4'	3 - 4'
14	3,5	3,5
15	4,4'	4 - 4'
Trichlorobiphenyls		
16	2,2',3	2,3 - 2'
17	2,2',4	2,4 - 2'
18	2,2',5	2,5 - 2'
19	2,2',6	2,6 - 2'
20	2,3,3'	2,3 - 3'
21	2,3,4	2,3,4
22	2,3,4'	2,3 - 4'
23	2,3,5	2,3,5
24	2,3,6	2,3,6
25	2,3',4	2,4 - 3'
26	2,3',5	2,5 - 3'
27	2,3',6	2,6 - 3'
28	2,4,4'	2,4 - 4'
29	2,4,5	2,4,5
30	2,4,6	2,4,6
31	2,4',5	2,5 - 4'
32	2,4',6	2,6 - 4'
33	2',3,4	3,4 - 2'
34	2',3,5	3,5 - 2'
35	3,3',4	3,4 - 3'
36	3,3',5	3,5 - 3'
37	3,4,4'	3,4 - 4'
38	3,4,5	3,4,5
39	3,4',5	3,5 - 4'
Tetrachlorobiphenyls		
40	2,2',3,3'	2,3 - 2',3'
41	2,2',3,4	2,3,4 - 2'
42	2,2',3,4'	2,3 - 2',4'
43	2,2',3,5	2,3,5 - 2'
44	2,2',3,5'	2,3 - 3',5'
45	2,2',3,6	2,3,6 - 2'
46	2,2',3,6'	2,3 - 2',6'
47	2,2',4,4'	2,4 - 2',4'
48	2,2',4,5	2,4,5 - 2'
49	2,2',4,5'	2,4 - 2',5'
50	2,2',4,6	2,4,6 - 2,
51	2,2',4,6'	2,4 - 2',6'
52	2,2',5,5'	2,5 - 2',5'
53	2,2',5,6'	2,5 - 2',6'
54	2,2',6,6'	2,6 - 2',6'
55	2,3,3',4	2,3,4 - 3'
56	2,3,3',4'	2,3 - 3',4'
57	2,3,3',5	2,3,5 - 3'
58	2,3,3',5'	2,3 - 3',5'
59	2,3,3',6	2,3,6 - 3'
60	2,3,4,4'	2,3,4 - 4'
61	2,3,4,5	2,3,4,5
62	2,3,4,6	2,3,4,6
63	2,3,4',5	2,3,5 - 4'
64	2,3,4',6	2,3,6 - 4'
65	2,3,5,6	2,3,5,6
66	2,3',4,4'	2,4 - 3',4'
67	2,3',4,5	2,4,5 - 3'
68	2,3',4,5'	2,4 - 3',5'
69	2,3',4,6	2,4,6 - 3'
70	2,3',4',5	2,5 - 3',4'
71	2,3',4',6	2,6 - 3',4'
72	2,3',5,5'	2,5 - 3',5'
73	2,3',5',6	2,6 - 3',5'
74	2,4,4',5	2,4,5 - 4'
75	2,4,4',6	2,4,6 - 4'
76	2',3,4,5	3,4,5 - 2'
77	3,3',4,4'	3,4 - 3',4'
78	3,3',4,5	3,4,5 - 3'
79	3,3',4,5'	3,4 - 3',5'
80	3,3',5,5'	3,5 - 3',5'
81	3,4,4',5	3,4,5 - 4'
Pentachlorobiphenyls		
82	2,2',3,3',4	2,3,4 - 2',3'
83	2,2',3,3',5	2,3,5 - 2',3'
84	2,2',3,3',6	2,3,6 - 2',3'
85	2,2',3,4,4'	2,3,4 - 2',4'
86	2,2',3,4,5	2,3,4,5 - 2'
87	2,2',3,4,5'	2,3,4 - 2',5'
88	2,2',3,4,6	2,3,4,6 - 2'
89	2,2',3,4,6'	2,3,4 - 2',6'
90	2,2',3,4',5	2,3,5 - 2',4'
91	2,2',3,4',6	2,3,6 - 2',4'
92	2,2',3,5,5'	2,3,5 - 2',5'
93	2,2',3,5,6	2,3,5,6 - 2'
94	2,2',3,5,6'	2,3,5 - 2',6'
95	2,2',3,5',6	2,3,6 - 2',5'
96	2,2',3,6,6'	2,3,6 - 2',6'
97	2,2',3',4,5	2,4,5 - 2',3'
98	2,2',3',4,6	2,4,6 - 2',3'
99	2,2',4,4',5	2,4,5 - 2',4'
100	2,2',4,4',6	2,4,6 - 2',4'
101	2,2',4,5,5'	2,4,5 - 2',5'
102	2,2',4,5,6'	2,4,5 - 2',6'
103	2,2',4,5',6	2,4,6 - 2',5'
104	2,2',4,6,6'	2,4,6 - 2',6'
105	2,3,3',4,4'	2,3,4 - 3',4'
106	2,3,3',4,5	2,3,4,5 - 3'
[redacted]		
110	2,3,3',4',6	2,3,6 - 3',4'
111	2,3,3',5,5'	2,3,5 - 3',5'
112	2,3,3',5,6	2,3,5,6 - 3'
113	2,3,3',5',6	2,3,6 - 3',5'
114	2,3,4,4',5	2,3,4,5 - 4'
115	2,3,4,4',6	2,3,4,6 - 4'
116	2,3,4,5,6	2,3,4,5,6
117	2,3,4',5,6	2,3,5,6 - 4'
118	2,3',4,4',5	2,4,5 - 3',4'
119	2,3',4,4',6	2,4,6 - 3',4'
120	2,3',4,5,5'	2,4,5 - 3',5'
121	2,3',4,5',6	2,4,6 - 3',5'
122	2',3,3',4,5	3,4,5 - 2',3'
123	2',3,4,4',5	3,4,5 - 2',4'
124	2',3,4,5,5'	3,4,5 - 2',5'
125	2',3,4,5,6'	3,4,5 - 2',6'
126	3,3',4,4',5	3,4,5 - 3',4'
127	3,3',4,5,5'	3,4,5 - 3',5'
Hexachlorobiphenyls		
128	2,2',3,3',4,4'	2,3,4 - 2',3',4'
129	2,2',3,3',4,5	2,3,4,5 - 2',3'
130	2,2',3,3',4,5'	2,3,4 - 2',3',5'
131	2,2',3,3',4,6	2,3,4,6 - 2',3'
132	2,2',3,3',4,6'	2,3,4 - 2',3',6'
133	2,2',3,3',5,5'	2,3,5 - 2',3',5'
134	2,2',3,3',5,6	2,3,5,6 - 2',3'
135	2,2',3,3',5,6'	2,3,5 - 2',3',6'
136	2,2',3,3',6,6'	2,3,6 - 2',3',6'
137	2,2',3,4,4',5	2,3,4,5 - 2',4'
138	2,2',3,4,4',5'	2,3,4 - 2',4',5'
139	2,2',3,4,4',6	2,3,4,6 - 2',4'
140	2,2',3,4,4',6'	2,3,4 - 2',4',6'
141	2,2',3,4,5,5'	2,3,4,5 - 2',5'
142	2,2',3,4,5,6	2,3,4,5,6 - 2'
143	2,2',3,4,5,6'	2,3,4,5 - 2',6'
144	2,2',3,4,5',6	2,3,4,6 - 2',5'
145	2,2',3,4,6,6'	2,3,4,6 - 2',6'
146	2,2',3,4',5,5'	2,3,5 - 2',4',5'
147	2,2',3,4',5,6	2,3,5,6 - 2',4'
148	2,2',3,4',5,6'	2,3,5 - 2',4',6'
149	2,2',3,4',5',6	2,3,6 - 2',4',5'
150	2,2',3,4',6,6'	2,3,6 - 2',4',6'
151	2,2',3,5,5',6	2,3,5,6 - 2',5'
152	2,2',3,5,6,6'	2,3,5,6 - 2',6'
153	2,2',4,4',5,5'	2,4,5 - 2',4',5'
154	2,2',4,4',5,6'	2,4,5 - 2',4',6'
155	2,2',4,4',6,6'	2,4,6 - 2',4',6'
156	2,3,3',4,4',5	2,3,4,5 - 3',4'
157	2,3,3',4,4',5'	2,3,4 - 3',4',5'
158	2,3,3',4,4',6	2,3,4,6 - 3',4'
159	2,3,3',4,5,5'	2,3,4,5 - 3',5'
160	2,3,3',4,5,6	2,3,4,5,6 - 3'
161	2,3,3',4,5',6	2,3,4,6 - 3',5'
162	2,3,3',4',5,5'	2,3,5 - 3',4',5'
163	2,3,3',4',5,6	2,3,5,6 - 3',4'
164	2,3,3',4',5',6	2,3,6 - 3',4',5'
165	2,3,3',5,5',6	2,3,5,6 - 3',5'
166	2,3,4,4',5,6,	2,3,4,5,6 - 4'
167	2,3',4,4',5,5'	2,4,5 - 3',4',5'
168	2,3',4,4',5,6	2,4,6 - 3',4',5'
169	3,3',4,4',5,5'	3,4,5 - 3',4',5'
Heptachlorobiphenyls		
170	2,2',3,3',4,4',5	2,3,4,5 - 2',3',4'
171	2,2',3,3',4,4',6	2,3,4,6 - 2',3',4'
172	2,2',3,3',4,5,5'	2,3,4,5 - 2',3',5'
173	2,2',3,3',4,5,6	2,3,4,5,6 - 2',3'
174	2,2',3,3',4,5,6'	2,3,4,5 - 2',3',6'
175	2,2',3,3',4,5',6	2,3,4,6 - 2',3',5'
176	2,2',3,3',4,6,6'	2,3,4,6 - 2',3',6'
177	2,2',3,3',4',5,6	2,3,5,6 - 2',3',4'
178	2,2',3,3',5,5',6	2,3,5,6 - 2',3',5'
179	2,2',3,4,4',5,5'	2,3,5,6 - 2',4',5'
180	2,2',3,4,4',5,5'	2,3,4,5 - 2',4',5'
181	2,2',3,4,4',5,6	2,3,4,5,6 - 2',4'
182	2,2',3,4,4',5,6'	2,3,4,5 - 2',4',6'
183	2,2',3,4,4',5',6	2,3,4,6 - 2',4',5'
184	2,2',3,4,4',6,6'	2,3,4,6 - 2',4',6'
185	2,2',3,4,5,5',6	2,3,4,5,6 - 2',5'
186	2,2',3,4,5,6,6'	2,3,4,5,6 - 2',6'
187	2,2',3,4',5,5',6	2,3,5,6 - 2',4',5'
188	2,2',3,4',5,6,6'	2,3,5,6 - 2',4',6'
189	2,3,3',4,4',5,5'	2,3,4,5 - 3',4',5'
190	2,3,3',4,4',5,6	2,3,4,5,6 - 3',4'
191	2,3,3',4,4',5',6	2,3,4,6 - 3',4',5'
192	2,3,3',4,5,5',6	2,3,4,5,6 - 3',5'
193	2,3,3',4',5,5',6	2,3,5,6 - 3',4',5'
Octachlorobiphenyls		
194	2,2',3,3',4,4',5,5'	2,3,4,5 - 2',3',4',5'
195	2,2',3,3',4,4',5,6	2,3,4,5,6 - 2',3',4'
196	2,2',3,3',4,4',5,6'	2,3,4,5 - 2',3',4',6'
197	2,2',3,3',4,4',6,6'	2,3,4,6 - 2',3',4',6'
198	2,2',3,3',4,5,5',6	2,3,4,5,6 - 2',3',5'
[redacted]		
202	2,2',3,3',5,5',6,6'	2,3,5,6 - 2',3',5',6'
203	2,2',3,4,4',5,5',6	2,3,4,5,6 - 2',4',5'
204	2,2',3,4,4',5,6,6'	2,3,4,5,6 - 2',4',6'
205	2,3,3',4,4',5,5',6	2,3,4,5,6 - 3',4',5'
Nonachlorobiphenyls		
206	2,2',3,3',4,4',5,5',6	2,3,4,5,6 - 2',3',4',5'
207	2,2',3,3',4,4',5,6,6'	2,3,4,5,6 - 2',3',4',6'
208	2,2',3,3',4,5,5',6,6'	2,3,4,5,6 - 2',3',5',6'
Decachlorobiphenyls		
209	2,2',3,3',4,4',5,5',6,6'	2,3,4,5,6 - 2',3',4',5',6'

Appendix Table 2 - PCB numbering scheme as described by Guitart *et al.* (2) and organized by ring chlorination pattern for easier identification of PCBs.

No.	Structure	Ring
Monochlorobiphenyls		
1	2	2
2	3	3
3	4	4
Dichlorobiphenyls		
4	2,2'	2 - 2
6	2,3'	2 - 3'
7	2,4'	2 - 4'
5	2,3	2,3
8	2,4	2,4
9	2,5	2,5
10	2,6	2,6
13	3,4'	3 - 4'
11	3,3'	3, 3'
12	3,4	3,4
14	3,5	3,5
15	4,4'	4 - 4'
Trichlorobiphenyls		
16	2,2',3	2,3 - 2'
20	2,3,3'	2,3 - 3'
22	2,3,4'	2,3 - 4'
21	2,3,4	2,3,4
23	2,3,5	2,3,5
24	2,3,6	2,3,6
17	2,2',4	2,4 - 2'
25	2,3',4	2,4 - 3'
28	2,4,4'	2,4 - 4'
29	2,4,5	2,4,5
30	2,4,6	2,4,6
18	2,2',5	2,5 - 2'
26	2,3',5	2,5 - 3'
31	2,4',5	2,5 - 4'
19	2,2',6	2,6 - 2'
27	2,3',6	2,6 - 3'
32	2,4',6	2,6 - 4'
33	2',3,4	3,4 - 2'
35	3,3',4	3,4 - 3'
37	3,4,4'	3,4 - 4'
38	3,4,5	3,4,5
34	2',3,5	3,5 - 2'
36	3,3',5	3,5 - 3'
39	3,4',5	3,5 - 4'
Tetrachlorobiphenyls		
40	2,2',3,3'	2,3 - 2',3'
42	2,2',3,4'	2,3 - 2',4'
46	2,2',3,6'	2,3 - 2',6'
56	2,3,3',4'	2,3 - 3',4'
44	2,2',3,5'	2,3 - 3',5'
58	2,3,3',5'	2,3 - 3',5'
41	2,2',3,4	2,3,4 - 2'
55	2,3,3',4	2,3,4 - 3'
60	2,3,4,4'	2,3,4 - 4'
61	2,3,4,5	2,3,4,5
62	2,3,4,6	2,3,4,6
43	2,2',3,5	2,3,5 - 2'
57	2,3,3',5	2,3,5 - 3'
63	2,3,4',5	2,3,5 - 4'
65	2,3,5,6	2,3,5,6
45	2,2',3,6	2,3,6 - 2'
59	2,3,3',6	2,3,6 - 3'
64	2,3,4',6	2,3,6 - 4'
47	2,2',4,4'	2,4 - 2',4'
49	2,2',4,5'	2,4 - 2',5'
51	2,2',4,6'	2,4 - 2',6'
66	2,3',4,4'	2,4 - 3',4'
68	2,3',4,5'	2,4 - 3',5'
48	2,2',4,5	2,4,5 - 2'
67	2,3',4,5	2,4,5 - 3'
74	2,4,4',5	2,4,5 - 4'
50	2,2',4,6	2,4,6 - 2,
69	2,3',4,6	2,4,6 - 3'
75	2,4,4',6	2,4,6 - 4'
52	2,2',5,5'	2,5 - 2',5'
53	2,2',5,6'	2,5 - 2',6'

No.	Structure	Ring
Tetrachlorobiphenyls (cont'd)		
72	2,3',5,5'	2,5 - 3',5'
70	2,3',4',5	2,5 - 3'4'
54	2,2',6,6'	2,6 - 2',6'
71	2,3',4',6	2,6 - 3',4'
73	2,3',5',6	2,6 - 3',5'
77	3,3',4,4'	3,4 - 3',4'
79	3,3',4,5'	3,4 - 3',5'
76	2',3,4,5	3,4,5 - 2'
78	3,3',4,5	3,4,5 - 3'
81	3,4,4',5	3,4,5 - 4'
80	3,3',5,5'	3,5 - 3',5'
Pentachlorobiphenyls		
82	2,2',3,3',4	2,3,4 - 2',3'
85	2,2',3,4,4'	2,3,4 - 2',4'
87	2,2',3,4,5'	2,3,4 - 2',5'
89	2,2',3,4,6'	2,3,4 - 2',6'
105	2,3,3',4,4'	2,3,4 - 3',4'
107	2,3,3',4,5'	2,3,4 - 3',5'
86	2,2',3,4,5	2,3,4,5 - 2'
106	2,3,3',4,5	2,3,4,5 - 3'
114	2,3,4,4',5	2,3,4,5 - 4'
116	2,3,4,5,6	2,3,4,5,6
88	2,2',3,4,6	2,3,4,6 - 2'
108	2,3,3',4,6	2,3,4,6 - 3'
115	2,3,4,4',6	2,3,4,6 - 4'
83	2,2',3,3',5	2,3,5 - 2',3'
98	2,2',3,4',5	2,3,5 - 2',4'
92	2,2',3,5,5'	2,3,5 - 2',5'
94	2,2',3,5,6'	2,3,5 - 2',6'
109	2,3,3',4',5	2,3,5 - 3',4'
111	2,3,3',5,5'	2,3,5 - 3',5'
93	2,2',3,5,6	2,3,5,6 - 2'
112	2,3,3',5,6	2,3,5,6 - 3'
117	2,3,4',5,6	2,3,5,6 - 4'
84	2,2',3,3',6	2,3,6 - 2',3'
91	2,2',3,4',6	2,3,6 - 2',4'
95	2,2',3,5',6	2,3,6 - 2',5'
96	2,2',3,6,6'	2,3,6 - 2',6'
110	2,3,3',4',6	2,3,6 - 3',4'
113	2,3,3',5',6	2,3,6 - 3',5'
97	2,2',3',4,5	2,4,5 - 2',3'
99	2,2',4,4',5	2,4,5 - 2',4'
101	2,2',4,5,5'	2,4,5 - 2',5'
102	2,2',4,5,6'	2,4,5 - 2',6'
119	2,3',4,4',5	2,4,5 - 3',4'
120	2,3',4,5,5'	2,4,5 - 3',5'
90	2,2',3',4,6	2,4,6 - 2',3'
100	2,2',4,4',6	2,4,6 - 2',4'
103	2,2',4,5',6	2,4,6 - 2',5'
104	2,2',4,6,6'	2,4,6 - 2',6'
119	2,3',4,4',6	2,4,6 - 3',4'
121	2,3',4,5',6	2,4,6 - 3',5'
122	2',3,3',4,5	3,4,5 - 2',3'
123	2',3,4,4',5	3,4,5 - 2',4'
124	2',3,4,5,5'	3,4,5 - 2',5'
125	2',3,4,5,6'	3,4,5 - 2',6'
126	3,3',4,4',5	3,4,5 - 3',4'
127	3,3',4,5,5'	3,4,5 - 3',5'
Hexachlorobiphenyls		
128	2,2',3,3',4,4'	2,3,4 - 2',3',4'
130	2,2',3,3',4,5'	2,3,4 - 2',3',5'
132	2,2',3,3',4,6'	2,3,4 - 2',3',6'
138	2,2',3,4,4',5'	2,3,4 - 2',4',5'
140	2,2',3,4,4',6'	2,3,4 - 2',4',6'
157	2,3,3',4,4',5'	2,3,4 - 3',4',5'
129	2,2',3,3',4,5	2,3,4,5 - 2',3'
137	2,2',3,4,4',5	2,3,4,5 - 2',4'
141	2,2',3,4,5,5'	2,3,4,5 - 2',5'
143	2,2',3,4,5,6'	2,3,4,5 - 2',6'
156	2,3,3',4,4',5	2,3,4,5 - 3',4'
159	2,3,3',4,5,5'	2,3,4,5 - 3',5'
142	2,2',3,4,5,6	2,3,4,5,6 - 2'
160	2,3,3',4,5,6	2,3,4,5,6 - 3'

No.	Structure	Ring
Hexachlorobiphenyls (cont'd)		
166	2,3,4,4',5,6,	2,3,4,5,6 - 4'
131	2,2',3,3',4,6	2,3,4,6 - 2',3'
139	2,2',3,4,4',6	2,3,4,6 - 2',4'
144	2,2',3,4,5',6	2,3,4,6 - 2',5'
145	2,2',3,4,6,6'	2,3,4,6 - 2',6'
158	2,3,3',4,4',6	2,3,4,6 - 3',4'
161	2,3,3',4,5',6	2,3,4,6 - 3',5'
133	2,2',3,3',5,5'	2,3,5 - 2',3',5'
135	2,2',3,3',5,6'	2,3,5 - 2',3',6'
146	2,2',3,4',5,5'	2,3,5 - 2',4',5'
148	2,2',3,4',5,6'	2,3,5 - 2',4',6'
162	2,3,3',4',5,5'	2,3,5 - 3',4',5'
134	2,2',3,3',5,6	2,3,5,6 - 2',3'
147	2,2',3,4',5,6	2,3,5,6 - 2',4'
151	2,2',3,5,5',6	2,3,5,6 - 2',5'
152	2,2',3,5,6,6'	2,3,5,6 - 2',6'
163	2,3,3',4',5,6	2,3,5,6 - 3',4'
165	2,3,3',5,5',6	2,3,5,6 - 3',5'
136	2,2',3,3',6,6'	2,3,6 - 2',3',6'
149	2,2',3,4',5',6	2,3,6 - 2',4',5'
150	2,2',3,4',6,6'	2,3,6 - 2',4',6'
164	2,3,3',4',5',6	2,3,6 - 3',4',5'
153	2,2',4,4',5,5'	2,4,5 - 2',4',5'
154	2,2',4,4',5,6'	2,4,5 - 2',4',6'
167	2,3',4,4',5,5'	2,4,5 - 3',4',5'
155	2,2',4,4',6,6'	2,4,6 - 2',4',6'
169	2,3',4,4',5',6	2,4,6 - 3',4',5'
169	3,3',4,4',5,5'	3,4,5 - 3',4',5'
Heptachlorobiphenyls		
170	2,2',3,3',4,4',5	2,3,4,5 - 2',3',4'
172	2,2',3,3',4,5,5'	2,3,4,5 - 2',3',5'
174	2,2',3,3',4,5,6'	2,3,4,5 - 2',3',6'
180	2,2',3,4,4',5,5'	2,3,4,5 - 2',4',5'
182	2,2',3,4,4',5,6'	2,3,4,5 - 2',4',6'
189	2,3,3',4,4',5,5'	2,3,4,5 - 3',4',5'
173	2,2',3,3',4,5,6	2,3,4,5,6 - 2',3'
181	2,2',3,4,4',5,6	2,3,4,5,6 - 2',4'
185	2,2',3,4,5,5',6	2,3,4,5,6 - 2',5'
186	2,2',3,4,5,6,6'	2,3,4,5,6 - 2',6'
190	2,3,3',4,4',5,6	2,3,4,5,6 - 3',4'
192	2,3,3',4,5,5',6	2,3,4,5,6 - 3',5'
171	2,2',3,3',4,4',6	2,3,4,6 - 2',3',4'
175	2,2',3,3',4,5',6	2,3,4,6 - 2',3',5'
176	2,2',3,3',4,6,6'	2,3,4,6 - 2',3',6'
183	2,2',3,4,4',5',6	2,3,4,6 - 2',4',5'
184	2,2',3,4,4',6,6'	2,3,4,6 - 2',4',6'
191	2,3,3',4,4',5',6	2,3,4,6 - 3',4',5'
177	2,2',3,3',4',5,6	2,3,5,6 - 2',3',4'
178	2,2',3,3',5,5',6	2,3,5,6 - 2',3',5'
179	2,2',3,4,4',5,5'	2,3,5,6 - 2',4',5'
187	2,2',3,4',5,5',6	2,3,5,6 - 2',4',5'
188	2,2',3,4',5,6,6'	2,3,5,6 - 2',4',6'
193	2,3,3',4',5,5',6	2,3,5,6 - 3',4',5'
Octachlorobiphenyls		
194	2,2',3,3',4,4',5,5'	2,3,4,5 - 2',3',4',5'
196	2,2',3,3',4,4',5,6'	2,3,4,5 - 2',3',4',6'
199	2,2',3,3',4,5,5',6'	2,3,4,5 - 2',3',5',6'
195	2,2',3,3',4,4',5,6	2,3,4,5,6 - 2',3',4'
198	2,2',3,3',4,5,5',6	2,3,4,5,6 - 2',3',5'
200	2,2',3,3',4,5,6,6'	2,3,4,5,6 - 2',3',6'
203	2,2',3,4,4',5,5',6	2,3,4,5,6 - 2',4',5'
204	2,2',3,4,4',5,6,6'	2,3,4,5,6 - 2',4',6'
205	2,3,3',4,4',5,5',6	2,3,4,5,6 - 3',4',5'
197	2,2',3,3',4,4',6,6'	2,3,4,6 - 2',3',4',6'
201	2,2',3,3',4,5',6,6'	2,3,4,6 - 2',3',5',6'
202	2,2',3,3',5,5',6,6'	2,3,5,6 - 2',3',5',6'
Nonachlorobiphenyls		
206	2,2',3,3',4,4',5,5',6	2,3,4,5,6 - 2',3',4',5'
207	2,2',3,3',4,4',5,6,6'	2,3,4,5,6 - 2',3',4',6'
208	2,2',3,3',4,5,5',6,6'	2,3,4,5,6 - 2',3',5',6'
Decachlorobiphenyls		
209	2,2',3,3',4,4',5,5',6,6'	2,3,4,5,6 - 2',3',4',5',6'